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Attorney's Docket No. ~~5051-587~~PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Gupta, et al.

Serial No.: 10/010,914

Filed: December 5, 2001

Group Art Unit: 1616

Examiner: F. Choi

For: *PHARMACEUTICAL COMPOSITIONS OF FENRETINIDE HAVING INCREASED BIOAVAILABILITY AND METHODS OF USING SAME*

September 29, 2004

Commissioner for Patents

P.O. Box 1450

Alexandria, Virginia 22313-1450

Rule 132 Declaration of Shanker Gupta

1. I, Shanker Gupta, do hereby declare and say as follows:
2. I am employed as a pharmacist in the Pharmaceutical Resources Branch of the National Cancer Institute of the National Institutes of Health. My education is set forth below:

<u>Date</u>	<u>Degree</u>	<u>Institute and Location</u>	<u>Field of Study</u>
1976	B.S	University of Florida	Pharmacy (Honors)
1982	Ph.D.	University of Michigan	Physical Pharmacy & Pharmaceutical Chemistry

My professional experience is set forth below:

- June 1995-Present: Pharmacist, Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health.
- October 1993-June 1995: Expert, Clinical Center Pharmacy Department, National Institutes of Health, Bethesda, Maryland.
- January 1987-October 1993: Senior Research Scientist (1987-1988), Research Investigator (1988-1993), PPD Liquid Products Development, Abbott Laboratories.

- April 1984-December 1986: Research Scientist, Pharmaceutical Development, Liquid Products, Travenol Laboratories, Inc. (Baxter Healthcare Corp.), Morton Grove, Illinois.
- 1982-1984: Research Investigator, Pharmaceutical Technology, Sterile, Semi-solids, and Liquids Sections, E.R. Squibb & Sons, New Brunswick, New Jersey.
- 1979-1982: Community Pharmacist, Maple Village Pharmacy, Ann Arbor, Michigan.

Some of my honors and awards are as set forth below:

- PHI KAPPA PHI
- RHO LAMBDA UPSILON (Chemistry Honor Society)
- RHO CHI (Pharmacy Honor Society)
- Who's Who Among Students in American Universities and Colleges, 1975-1976

My research interests are as follows:

- Surfactant chemistry in pharmaceutical formulations
- Use of parenteral emulsions as drug carrier systems
- Aqueous solution kinetics

Some of my publications are as follows:

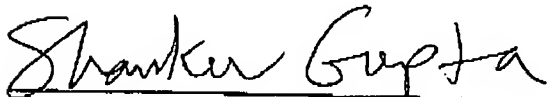
- V. K. Sarin, S. Gupta, K. Leung, V. Taylor, B.L. Ohning, J. A. Whitsett, and L. J. Fox. "Biophysical and Biological Activity of a Synthetic 8.7 kSa Hydrophobic Pulmonary Surfactant Protein SP-B." Proc. Nat. Acad. Sci. USA, 87: 2633-2637, 1990.
- Steven L. Krill and Shanker L. Gupta. "Effect of a Bovine Lung Surfactant Isolate (SP-B/C) on Egg Phosphatidylglycerol Acyl Chain Order in a Lipid Mixture with Dipalmitoylphosphatidylcholine and Palmitic Acid." J. Pharm. Sci., 83: 539-541, 1994.
- Steven L. Krill, Shanker L. Gupta, and Tricia Smith. "Pulmonary Lung Surfactant Synthetic Peptide Concentration Dependent Modulation of DPPC and POPG Acyl Chain Order in a DPPC:POPG:Palmitic Acid Lipid Mixture." Chem. Phys. Lipids, 71: 47-59 (1994).

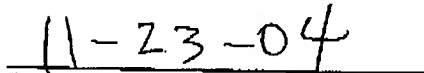
- **Shanker L. Gupta, Jitendra P. Patel, David L. Jones, and Raymond W. Partipilo.** "Preclinical Development and Characterization of an Intravenous Dosage Form for the Renin Inhibitor Abbott-72517." J. Parenter. Sci. Technol., 48: 86-91, 1994.

Ex. 1, Curriculum Vitae of Shanker L. Gupta.

3. I am a co-inventor on the above-captioned patent application and have read the comments of the Examiner thereon.
4. It is my understanding that the Examiner has rejected claims 11-41 because the specification only discloses non-ionic egg phospholipids, which the Examiner maintains are non-enabled because the prior art indicates that egg phospholipids are ionic surfactants.
5. However, the biochemistry of egg phospholipids shows that it is known for one in the field of pharmaceutical formulation chemistry to use egg phospholipids as non-ionic surfactants, and to understand that, contrary to what the Examiner asserts, egg phospholipids can be used as non-ionic surfactants.
6. Some of the confusion regarding the use of egg phospholipids as non-ionic surfactants arises because a phospholipid can be an ionic surfactant under some conditions, but can also be non-ionic under other conditions. This is because the phospholipid's net charge depends on pH. See Ex. 2, Mustafa Grit and Daan J. A. Crommelin. "Chemical Stability of Liposomes: Implications for Their Physical Stability." Chem. Phys. Lipids, 64: 3-18, 1993.
7. The egg phospholipids described in the above-captioned application were non-ionic because they were utilized as neutral molecules at physiological pH.
8. For example, one common commercially available egg phospholipid is Lipoid E 80. See Ex. 3, U.S. Patent No. 6,228,399 at 3:14-16. It is known that Lipoid E 80 contains approximately 82% egg phosphatidylcholine (PC) and 8% egg phosphatidylethanolamine (PE). Both are neutral at physiological pH by virtue of the fact that they possess a quaternary ammonium group, which is positively charged, and a negative charge on the phosphate group such that it will be negatively charged at a pH of about 3.5 or greater. See Ex. 2 at p. 8.

9. Egg phospholipids have been utilized by others in the field of pharmaceutical formulation chemistry and referred to as a nonionic surfactant. See Ex. 4, Timothy J. Young, Keith P. Johnston, Gary W. Pace, and Awadhesh K. Mishra. "Phospholipid-Stabilized Nanoparticles of Cyclosporine A by Rapid Expansion from Supercritical to Aqueous Solution." AAPS PharmSciTech, 5 (1) Article 11 (<http://www.aapharmstech.org>), 2003 (describing solution as including nonionic surfactants (p. 1) and listing Lipoid E80 as one of the surfactants used (p. 5)).
10. Because (a) egg phospholipids are common materials; (b) the constituents of egg phospholipids are known to contain a quaternary ammonium group; (c) such phospholipids containing a quaternary ammonium group are neutral at physiological pH; and (d) because other skilled persons in the field have described egg phospholipids as nonionic surfactants, persons skilled in the art would easily be able to practice my invention utilizing egg phospholipids as nonionic surfactants.
11. All of the statements made above of the undersigned declarant's own knowledge are true and all statements made on information and belief are believed to be true. The undersigned acknowledges that any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. §1001), and may jeopardize the validity of the above-referenced application or any patent issuing thereof.


Dr. Shanker Gupta


Date

Enclosures:

- Ex. 1, Curriculum Vitae of Shanker L. Gupta;
Ex. 2, Mustafa Grit and Daan J. A. Crommelin. "Chemical Stability of Liposomes: Implications for Their Physical Stability," Chem. Phys. Lipids, 64: 3-18, 1993.;
Ex. 3, U.S. Patent No. 6,228,399 at 3:14-16;
Ex. 4, Timothy J. Young, Keith P. Johnston, Gary W. Pace, and Awadhesh K. Mishra. "Phospholipid-Stabilized Nanoparticles of Cyclosporine A by Rapid Expansion from Supercritical to Aqueous Solution." AAPS PharmSciTech, 5 (1) Article 11 (<http://www.aapharmstech.org>), 2003.

SHANKER L. GUPTA

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EDUCATION

University of Florida
B.S. Pharmacy (Honors), 1976

University of Michigan
Ph.D., Physical Pharmacy & Pharmaceutical Chemistry, 1982
Major Professor: Dr. William I. Higuchi
Thesis Title: "Mechanistic Study of Cholesterol Monohydrate Dissolution in Aqueous Micellar Surfactant and Sodium Chenodeoxycholate Solutions"

HONORS

Who's Who Among Students in American Universities & Colleges, 1975-1976
RHO CHI (Pharmacy Honor Society)
RHO LAMBDA UPSILON (Chemistry Honor Society)
PHI KAPPA PHI

PROFESSIONAL EXPERIENCE

National Institutes of Health

Pharmaceutical Resources Branch

Division of Cancer Treatment
National Cancer Institute
Pharmacist
June, 1995 - Present

Responsibilities

- Manage and coordinate contracts for therapeutic dosage form development and production of anti-neoplastic compounds.
- Collaborate with R & D Contractor in the strategic development of dosage forms for sparingly soluble cytotoxic agents.
- Schedule laboratory studies and production schedule based on the Division priorities at the R & D Contractors site.

- Manufacture/schedule clinical batches per cGMPs based on Division priorities.
- File CMC section of Investigational New Drug Applications with Regulatory Affairs Branch for submission to FDA.
- Schedule/set up/monitor clinical product shelf life.
- Interface with other intramural laboratories and extramural branches for timely conductance of research projects.
- Interface with the Biological Resources Branch in the development and production of biological drugs.

Research Activities & Accomplishments

- Preformulation and formulation development of several peptides leading to stable dosage form - ras (cys), gp 100 peptides, MART-1, CAP-1-6D.
- Stable dosage form for several cytotoxic agents – NSC 676418, Pc4; NSC 681239D, peptidic boronic acid.
- IND submission.
- 1 patent, 1 book chapter, 1 publication, 8 presentations.

Clinical Center Pharmacy Department

Expert

October, 1993 - June, 1995

Responsibilities

- Assist in a review, audit, qualification and validation of equipment, processing systems, and management of Pharmaceutical Development Section's new sterile products pilot scale production facility for biotech products so that the facility conforms to and meets with FDA approval as a current Good Manufacturing Practice (cGMP) site.
- Review the facility from a viewpoint of manufacturing biotech related products such as monoclonal antibodies, vaccines, recombinant protein products to insure that the facility meets FDA regulations governing biotech pilot scale facilities.
- Serve as an advisor to the Department in the areas of formulation, processes and equipment pertaining to biotech products.

Research Activities

- Conducted preformulation, formulation development, and manufacture of ras peptides clinical product to be used as vaccines.
- Mentor to the Pharmacy Practice resident in a study on the conformation stability of interleukin-2 (IL-2) in the presence of secondary drugs commonly used at the Clinical Center. The compatibility studies of protein drugs require examination of their secondary structure using biochemical techniques such as circular dichroism(CD). CD was successful used to assess the conformation stability of IL-2.
- Characterization and formulation development of Malaria Vaccine. The bulk antigen was recombinantly produced. We studied its stability, and secondary structure. This was accomplished through the use of circular dichroism, total protein assay and HPLC.

Abbott Laboratories

PPD Liquid Products Development

January, 1987 - October, 1993

Research Investigator

1988 - 1993

Responsibilities

- Performed formulation development studies for a natural pulmonary surfactant resulting in a marketed product.
- Studied role of synthetic surfactant proteins in various formulation matrices leading to two patents.
- Preformulation and formulation development studies for two new chemical entities including a lyophilized product for Phase I clinical trials.
- Managed a team of four associate scientists.
- Project leader for the pulmonary surfactant project.
- Interfaced with QA, QC, Manufacturing during Development and for the Product Launch across four major divisions of Abbott Laboratories.
- Provided NDA, IND reports to Drug Regulatory Affairs Department.

- Pre-clinical and clinical development support to the Discovery Scientists.

Research Activities

- Collaborated with discovery scientists to identify bioactive fragments of pulmonary surfactant protein B based on SAR studies and surfactant protein C conjugate.
- Studied pulmonary surfactant protein-lipid interactions using biophysical techniques.
- Studied alternate methods of formulation for sparingly soluble compounds.
- Conducted experiments to predict irritation at the site of injection.
- Published several internal reports on preformulation, formulation development.
- Published several journal articles and made presentations at National Professional Meetings.
- Reviewer for a national pharmaceutical journal.

PPD Liquid Products Development
Senior Research Scientist
1987 - 1988

Responsibilities

- Initiated product development effort for both synthetic as well as natural lung surfactant.
- Promoted to Research Investigator position.

Chemical stability of liposomes: implications for their physical stability

Mustafa Grit* and Daan J.A. Crommelin

Department of Pharmaceutics, Faculty of Pharmacy, University of Utrecht P.O. Box 80082, 3508 TB Utrecht (The Netherlands)

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In the first part of this article, chemical and physical stability of aqueous liposome dispersions have been addressed. Chemical stability of phospholipids has been considered in two parts: oxidation and hydrolysis. Major attention has been paid to hydrolysis kinetics of phospholipids as a function of pH, temperature, buffer concentration and ionic strength. Furthermore, the effect of chain length, head group, state of aggregation, addition of cholesterol and presence of charge on the hydrolysis kinetics of phospholipids has been dealt with. In the second part physical stability of chemically degraded liposome dispersions has been evaluated. In the final part quality control assays for liposome dispersions is presented and a HPLC method with a refractive index detector for the analysis of phospholipids from aqueous liposome dispersions is described.

Key words: liposomes; phospholipids; oxidation; hydrolysis; physical stability; quality control; HPLC analysis of phospholipids

Introduction

In the last two decades, it has been demonstrated that encapsulation of drugs into liposomes can lead to the enhancement of therapeutic efficacy of drugs, reduction of their toxicity and prolongation of their therapeutic effect. In addition, studies have been performed on the use of liposome-based vaccines. Recently, a liposome formulation containing the antifungal agent amphotericin has been approved by regulatory authorities and is presently used in the clinic. From a pharmaceutical point of view, drugs and dosage forms must be sufficiently stable to be stored for at least 1 year and preferably longer periods of time. Liposomal drug formulations have to comply with this rule.

In this article liposomal stability issues are addressed. The chemical stability of phospholipids

is dealt with first. The main focus is on phosphatidylcholine as it is most commonly used in pharmaceutical liposome preparations. The physical stability of liposome dispersions and the relationship between physical and chemical stability of liposomes are the subjects of interest in the second part. Finally, attention is paid to quality control aspects of liposome dispersions.

Chemical stability of phospholipids

Liposome-forming phospholipids are obtained from natural sources or through synthetic routes. Natural phospholipids are not easy to characterise in terms of their exact phospholipid composition. In contrast, synthetic phospholipids can be well characterised and can be obtained in a highly purified form. The chemical structure of a number of phospholipids regularly used for liposome preparations is shown in Fig. 1.

Two degradation pathways, which might limit the shelf life of liposome dispersions, have been described for phospholipids in (aqueous) liposome dispersions. These are the oxidative and the hydrolytic degradation pathways.

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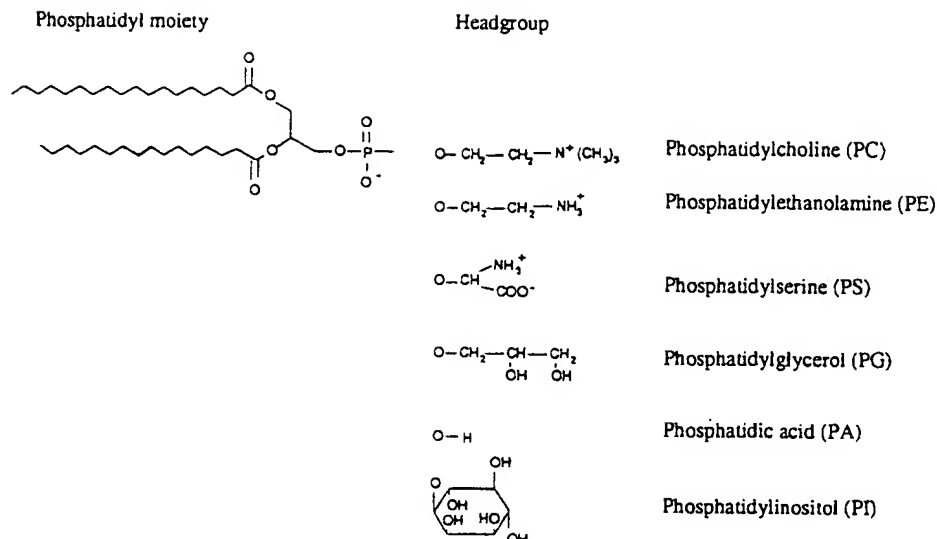


Fig. 1. Structure of a number of phospholipids regularly used in liposomes [5].

Oxidation of phospholipids

Oxidation of phospholipids, particularly those with unsaturated fatty acyl chains, has obtained considerable attention throughout the years and was extensively reviewed [1–4]. Oxidation of phospholipids in liposomes mainly takes place in unsaturated fatty acyl chain-carrying phospholipids. But, saturated fatty acids can also be oxidized at high temperatures [1]. The fatty acyl chains of phospholipid molecules are oxidized via a free radical chain mechanism in the absence of specific oxidants. Exposure to electromagnetic radiation and/or the presence of trace amounts of transition metal ions initiate radical formation (hydrogen atom abstraction) in the lipid chain (initial step). Since unsaturation permits delocalization of the remaining unpaired electron along the lipid chain, lipids containing polyunsaturated fatty acids are the most sensitive to radical formation. If oxygen is present, the process develops further and via the formation of hydroperoxides fission of the fatty acid chain can occur [5].

Peroxidation of phospholipids in liposomes can be minimised by the use of high quality raw mate-

rials which are purified from hydroperoxides and transition metal ions. Storage at low temperatures, protection from light and oxygen will exclude the chance of oxidation. To further enhance protection against oxidation, antioxidants, such as α -tocopherol and butyl hydroxy toluene (BHT) can be added. Moreover, EDTA can be used to form a complex with transition metal ions. Working under an inert gas atmosphere such as nitrogen or argon reduces the oxidation of lipids during preparation of liposomes. Recently, a protective effect of buffers such as HEPES and Tris against lipid peroxidation has been reported by Fiorentini et al. [6]. Oxidation of phospholipids can also be overcome by using less unsaturated fatty acyl chain-containing phospholipids. Lang et al. [7] investigated the sensitivity of a series of (partially hydrogenated) egg phosphatidylcholine (PHEPC) molecules against accelerated oxidation in the presence of 2,2'-azobis-(2-amidinopropane)hydrochloride (AAPH). They observed a decreased sensitivity of (PH)EPC with a decreasing degree of unsaturation (Fig. 2). The use of partially hydrogenated phospholipids might be preferred over natural phospholipids, since up to

a certain hydrogenation level partially hydrogenated lipids in aqueous dispersions do not show a phase transition above 0°C that physically disturbs the bilayer structure while the sensitivity to oxidation decreases substantially [7].

Hydrolysis of phospholipids in aqueous liposome dispersions

Hydrolysis products and reaction scheme

The four ester bonds present in a phospholipid molecule may be subject to hydrolysis in water (Figs. 1 and 3). The carboxy esters at *sn*-1 and *sn*-2 positions are hydrolysed faster than the phosphate esters [8]. Hydrolysis of esters is catalysed in the presence of acid and base. In the acid-catalysed hydrolysis of esters, the ester is first protonated and subsequently a nucleophilic attack of water takes place (rate determining step). The base-catalysed hydrolysis of an ester proceeds via an addition-elimination mechanism. Here, the rate determining step is the attack of hydroxide ions on the carbonyl carbon atom first. In both cases, fast addition-elimination reactions follow the rate determining steps [8]. This process can take place

if the esters are hydrated. In a phospholipid bilayer in an aqueous medium the position of the hydrophobic/hydrophilic boundary is, on the average, positioned at the level of the first CH₂ group in the hydrocarbon chains [9]. These considerations are based on calculations of the dimensions of the hydrophilic and hydrophobic parts of PC and PE bilayers as derived from X-ray long spacing data. This finding indicates that water and consequently, proton and hydroxide ions have access to all ester bonds.

2-Acyl and 1-acyl lysophospholipids are formed as a result of hydrolysis of the carboxy esters at the *sn*-1 and *sn*-2 positions, respectively. Further hydrolysis of both lysophospholipids produces a glycerophospho compound. Glycerophosphoric acid, the end hydrolysis product, is produced by hydrolysis of the phosphate-headgroup (e.g. choline, ethanolamine, serine) ester. The hydrolysis of the ester bond between glycerol and phosphoric acid appears to be very slow and, therefore, no free phosphoric acid and glycerol are produced under pharmaceutically relevant conditions [10,11]. In Fig. 3, hydrolysis and acyl migration reactions which can take place in phospholipids in aqueous liposome dispersions are summarised; PC is taken as an example.

Under both acidic and alkaline conditions hydrolysis of PC in aqueous liposome dispersions follows pseudo first-order kinetics [12–16]. Upon analysis the major initial hydrolysis product is 1-acyl lysophosphatidylcholine (LPC) (erroneously often called 2-LPC) [12,13,15–17]. If the hydrolysis reactions are not monitored in detail for both positions, the overall results obtained give the impression that hydrolysis mainly takes place at the *sn*-2 position. However, hydrolysis takes place at both positions. 2-Acyl lysophospholipids are rapidly converted to 1-acyl lysophospholipids via an acyl migration reaction. 1-Acyl lysophospholipids are the most stable form of the two isomeric lysophospholipids [18]. Acyl migration reactions are pH-dependent and a minimum rate of acyl migration can be found around pH 4.0–5.0; acyl migration reactions tend to be catalysed by buffer species.

Kensil and Dennis determined the hydrolysis

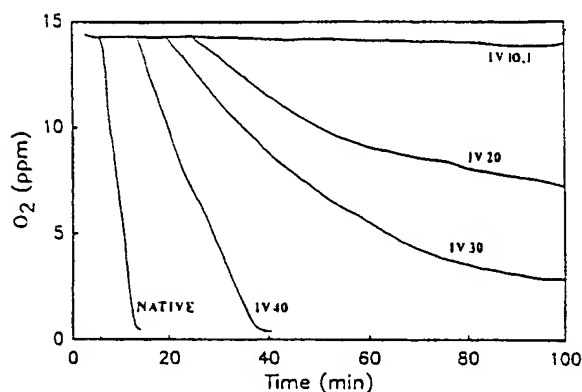


Fig. 2. Oxygen uptake of liposomes prepared from a series of (partially hydrogenated) egg PC molecules during AAPH-stimulated oxidation in small unilamellar vesicles (SUV). Plot shows oxygen uptake in dispersions of SUV in closed stirred vessels at room temperature as measured by an oxygen electrode. AAPH was added at time 0. Iodine values (IV), which are a measure of the degree of unsaturation, are given in the plot. Data from Ref. 7.

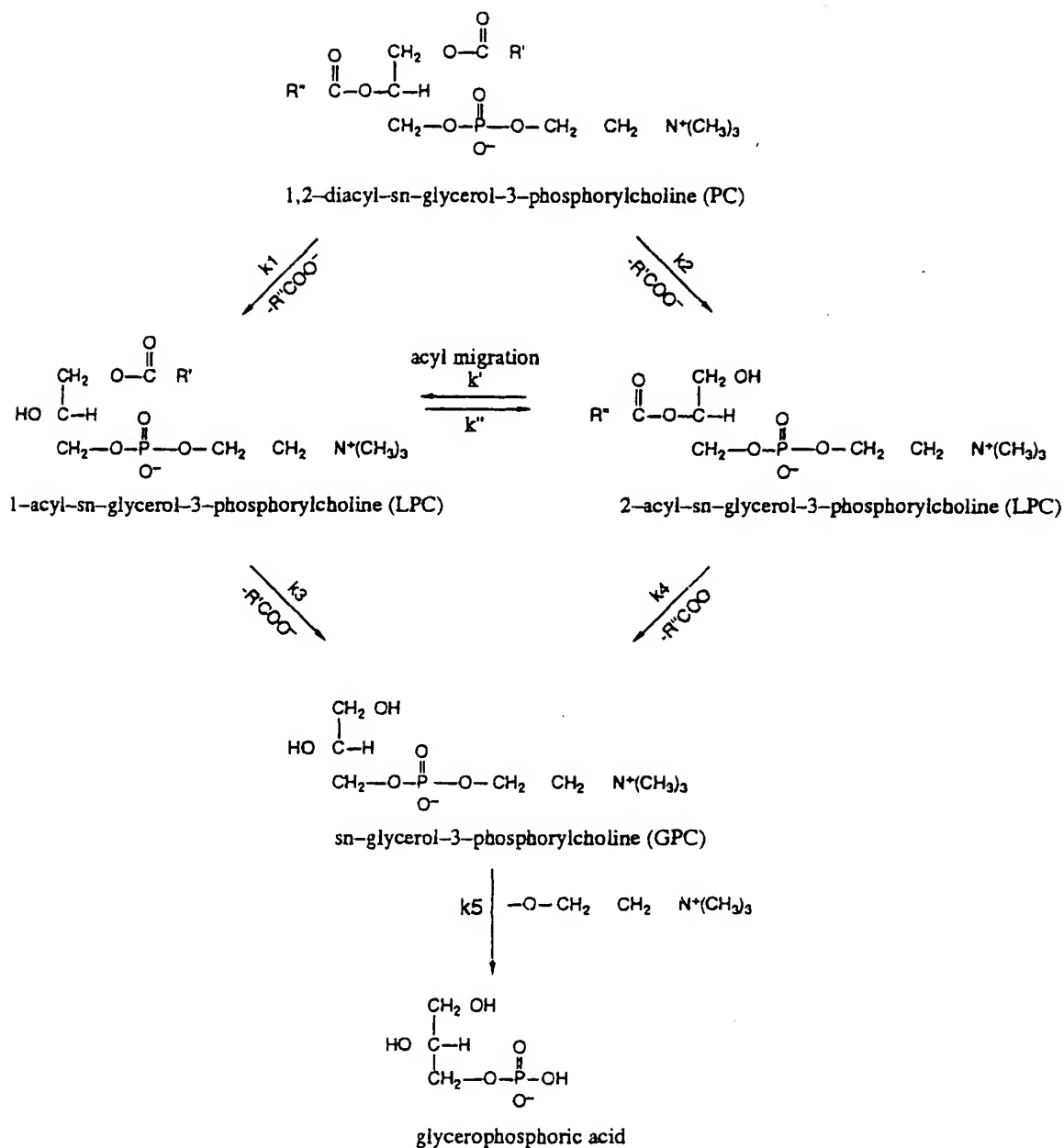


Fig. 3. Hydrolysis reactions of PC in aqueous liposome dispersions. R' and R'' are acyl chains.

rate constants at the *sn*-1 and *sn*-2 positions of dipalmitoyl phosphatidylcholine (DPPC) with ^{14}C labelled preferentially in the *sn*-2 fatty acid chain in a liposome dispersion and in Triton X-100/PC mixed micelles at a molar ratio of 4/1 at 40 and 50°C and at the rather exotic pH 12.7 by determin-

ation of the radioactivity of the formed LPC [12]. They found equal hydrolysis rates at both positions over the first half-life of the hydrolysis process. The hydrolysis of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) under extreme acid conditions (pH 1.0) was studied by Ho et al. [19]. The

formation of palmitic and oleic acid from the *sn*-1 and *sn*-2 positions, respectively, was followed in time. No significant differences were found between the concentrations of oleic and palmitic acid in time over the first part of the hydrolysis process. Similar results were found in a study where the hydrolysis kinetics of saturated soybean PC were investigated [15].

The reaction time course of the hydrolysis of saturated soybean PC in 0.05 M acetate buffer (pH 4.0) at 70°C is presented in Fig. 4. The concentration patterns of PC, free fatty acids, the two lysophosphatidylcholines and glycerophosphocholine as a function of time are plotted, providing a detailed picture of the hydrolysis process. The formation of lysophospholipids is often taken as a measure for hydrolysis of phospholipids in liposome dispersions in studies on the chemical stability of phospholipids [13,20,21]. As shown clearly in Figs. 3 and 4, lysophosphatidylcholines are intermediate hydrolysis products. Hydrolysis kinetics of phospholipids, therefore, cannot be monitored by following the lysophospholipid concentration in liposome dispersions. The conversion rate of lysophospholipids to their glycerophospho analog is not exactly known yet. In some studies it was reported to be faster than that of the hydrolysis of phospholipids to lysophospholipids [12,19].

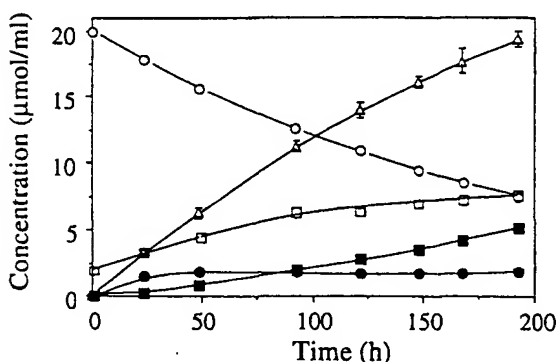


Fig. 4. Disappearance of saturated soybean PC and the appearance of hydrolysis products of PC in pH 4.0 acetate buffer ($C = 0.05$ M) at 70°C. Data from Ref. 15. $n = 12$; bars indicate standard errors. O, Phosphatidylcholine (PC); ●, 2-acyl-lysophosphatidylcholine (*sn*-2 LPC); □, 1-acyl-lysophosphatidylcholine (*sn*-1 LPC); ■, glycerophosphocholine (GPC); Δ, free fatty acids.

The effect of pH

Detailed studies on the effect of pH on the hydrolysis of saturated soybean PC in a pH range from 4.0–9.0 at 40°C and 70°C were published by Grit et al. [15]. As shown in Fig. 5 the hydrolysis rate of saturated soybean PC reached a minimum at pH 6.5 and V-shaped pH profiles were obtained as expected for an acid/base-catalysed ester hydrolysis. Similar results were reported for the hydrolysis of distearoyl phosphatidylcholine (DSPC), natural soybean PC and partially hydrogenated egg PC [13,14,16].

The effect of temperature

The effect of temperature on the hydrolysis of phospholipids has been studied with synthetic and/or natural PC. For all PC's the results could be described by Arrhenius kinetics as a semi-logarithmic linear relationship was found between the observed hydrolysis rate constant and the reciprocal of the absolute temperature. For natural PC's Arrhenius kinetics held in a temperature range from room temperature up to 82°C [14,16]. However, for phospholipids composed of saturated fatty acids and with a liquid to gel crystalline phase transition due to melting of the fatty acyl chains, biphasic Arrhenius curves were obtained with a discontinuity around the transition temperature [12,13,15]. Arrhenius plots for the hydrolysis of saturated soybean PC and partially hydro-

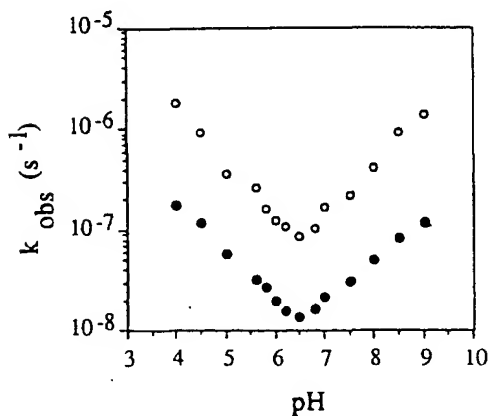


Fig. 5. The effect of pH on the hydrolysis of saturated soybean PC at 40°C (●) and 70°C (○). Buffer concentration = 0.05 M. Data from Ref. 15.

generated egg PC are presented in Fig. 6. Frøkjær found a higher activation energy in DSPC liposomes for pH 6.5 for bilayers in the gel state than in the liquid crystalline state [13]. No information was given about the confidence intervals for the published data. The difference between the two activation energies may not be statistically significant, when the standard errors for the observed hydrolysis rate constants are taken into account. With DPPC liposomes Kensil and Dennis found the same activation energy in bilayers in both the gel and the liquid crystalline phase at pH 12.7 [12]. Furthermore, the jump in the observed rate constants was less pronounced for the DPPC liposomes than for the DSPC liposomes. For saturated soybean PC, the activation energies below and above the transition temperature were found to be similar if the (expected) standard deviations are taken into account [15].

The effect of ionic strength

The effect of ionic strength on the hydrolysis kinetics of partially hydrogenated egg PC was studied at pH 4.5, 6.5 and 8.0 at 70°C [16]. No effect of the ionic strength on the hydrolysis kinetics was observed. Theoretically, the ionic strength affects the reaction kinetics only if the reaction takes place between two charged species. As PC has no net charge at the studied pH values, no ionic strength effect on the hydrolysis kinetics was expected and the results confirmed this prediction.

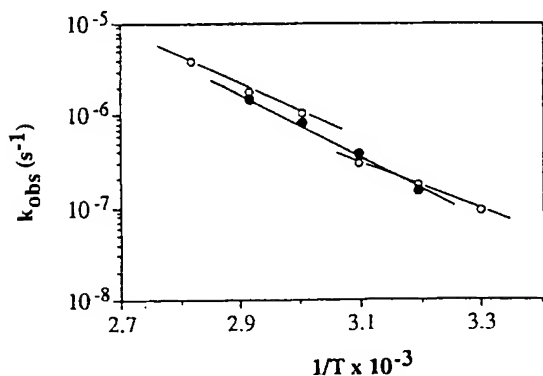


Fig. 6. The effect of temperature on the hydrolysis of saturated soybean PC (O) and partially hydrogenated egg PC (●) in pH 4.0 acetate buffer ($C = 0.05$ M). Data from Refs. 15 and 16.

The effect of buffer species

The effect of buffer species on the hydrolysis process has recently been studied for the first time in different buffer solutions for natural soybean PC, saturated soybean PC and partially hydrogenated egg PC [14–16]. The formation of fatty acids upon the hydrolysis of fatty acyl esters tends to change the pH during storage. In order to be able to obtain iso-pH hydrolysis rate constants, the pH of the dispersions must be kept constant over the experimental period by using appropriate buffer solutions. Then, the possible catalytic effect of the buffer species on the hydrolysis reactions has to be taken into account [22]. To quantitate the contribution of the buffer species on hydrolysis kinetics, the experiments were performed at different buffer concentrations, for different pH values with the same buffer at constant temperature. Detailed information on the quantitative analysis of catalysed hydrolysis data and calculation of the second-order rate constants have been published elsewhere [14]. Figure 7 shows the effect of buffer concentration on the hydrolysis rate constant of partially hydrogenated egg PC in HEPES buffer for different pH values at 70°C [16]. The second-order rate constants for the catalysed hydrolysis of natural soybean PC, saturated soybean PC and partially hydrogenated egg PC by different buffer species are listed in Table I [14–16]. In general, buffer species catalyse the hydrolysis process. When the second-order rate constants for the catalysed hydrolysis are compared and when the regularly used buffer concen-

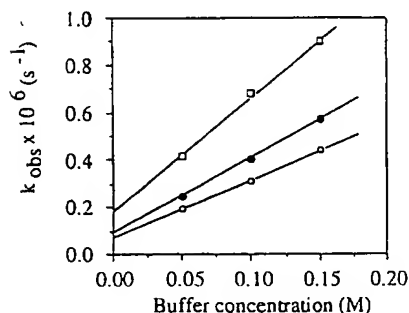


Fig. 7. The effect of buffer concentration on the hydrolysis of partially hydrogenated egg PC in HEPES buffers at 70°C. Data from Ref. 16. O, pH 6.8; ●, pH 7.0; □, pH 7.5

TABLE I

Second-order rate constants for the catalysed hydrolysis of PC at 70°C.

	Natural soybean PC ^a	Saturated soybean PC ^b	Partially hydrogenated egg PC ^c
k_0	2.0×10^{-7}	1.0×10^{-8}	6.2×10^{-8}
k_H	2.2×10^{-2}	2.4×10^{-2}	1.6×10^{-2}
k_{OH}	1.9×10^{-1}	1.2×10^{-1}	2.7×10^{-1}
k_{Ac^-}	5.5×10^{-6}	1.7×10^{-5}	1.1×10^{-5}
k_{HAc}	-1.6×10^{-6}	-2.5×10^{-6}	-1.6×10^{-6}
k_{Tris^+}	1.0×10^{-5}	8.9×10^{-6}	7.9×10^{-6}
k_{Tris}	1.1×10^{-6}	8.9×10^{-7}	8.7×10^{-7}

Rate constants are expressed in $M^{-1} s^{-1}$, except for the first-order rate constant k_0 which is reported in s^{-1} .^aTaken from Ref. 14.^bTaken from Ref. 15.^cTaken from Ref. 16.

trations are considered, it can be concluded that under pharmaceutically relevant conditions the hydrolysis is mainly catalysed by protons and hydroxyl ions.

The effect of chain length and headgroup

No systematic studies have been performed on the effect of chain length and headgroup on the hydrolysis of phospholipids in liposome dispersions. The rate constants of the alkaline hydrolysis of synthetic PC's with various chain lengths, egg PC and egg PE obtained at 40°C and pH 12.7 in a mixed micellar solution of Triton X-100/phospholipid in a molar ratio of 8/1 are listed in Table II [12]. The authors state that the differences be-

tween the data sets were found to be significant when analysed with a Student's *t*-test. The data suggest that as the chain length of these neutral phospholipids decreases, the rate of hydrolysis tends to increase and that the degree of saturation of the acyl chains also affects the rate. The mechanism behind these observations is not fully understood yet. In a study where the hydrolysis kinetics of partially hydrogenated, neutral egg PC and negatively charged egg PG were studied in liposomes with PHEPC/EPG = 10/1 bilayers, it was found that egg PG hydrolyses slightly faster than partially hydrogenated egg PC [23]. The difference found could possibly be ascribed to the difference in degree of unsaturation of the phospholipids studied and/or the presence of the net charge.

TABLE II^a

The effect of chain length and headgroup on the hydrolysis rate constant (k_{obs}) of phospholipids in mixed micellar solution of Triton X-100/phospholipid (molar ratio 8/1) at 40°C and at pH 12.7.

Phospholipid	$k_{obs} \times 10^3 (M^{-1} \cdot s^{-1})$
DLPC	16.0
DMPC	15.0
DPPC	13.2
Egg PC	14.1
Egg PE	4.6

^aData from Ref. 12.

The effect of the state of aggregation

If the accessibility of the esters in the phospholipid molecule by water and, consequently, proton and hydroxyl ions is affected by the aggregation state of the phospholipids, differences could be expected between hydrolysis rate constants of phospholipids in different aggregation states. Kensil and Dennis [12] determined the hydrolysis rate constants of egg PC and DPPC in liposomes and in mixed micelles (prepared with Triton X-100). Both egg PC and DPPC hydrolyse faster in mixed micelles than in liposomes. Hydrolysis rate con-

stants of egg PC in liposomes and in mixed micelles at 40°C and at pH 12.7 were $1.4 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ and $14.1 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$, respectively. The rate constants for DPPC were $0.7 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ in liposomes and $13.8 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ in mixed micelles. Thus, under those conditions the rate constants in vesicles were 10–20-fold lower than those in mixed micelles. Moreover, the activation energies of both egg PC and DPPC were lower in mixed micelles than in vesicles. The authors conclude that the aggregation state strongly influences hydrolysis kinetics presumably by affecting the interfacial region between the lipid molecules and water [12].

The effect of cholesterol incorporation in the bilayers on the hydrolysis kinetics of phosphatidylcholine

The incorporation of cholesterol into liposomal bilayers tends to increase the retention of hydrophilic drugs, counteracts lipid phase transition and increases the rigidity of fluid state bilayers and thereby the resistance to in vivo liposome degradation. This explains why cholesterol is often a part of pharmaceutical liposome formulations [24–27].

Different interaction mechanisms between bilayer-forming phospholipids and cholesterol have been discussed. The formation of hydrogen bonds between the 3-OH group of cholesterol and the carbonyl group of the fatty acyl ester bonds of phospholipids at both *sn*-1 and *sn*-2 positions has been proposed [28,29]. Depending on the strength of this interaction, the hydration properties of both ester bonds may be changed. Any change in the hydration properties of the esters can affect the hydrolysis kinetics, if access of water molecules to the ester bond is a critical factor. This effect is, in general, expected to be a protective effect. Certain properties of the bilayers formed with a mixture of cholesterol and either a di-ester GPC or a di-ether GPC, which is lacking ester carbonyl groups, have been reported [30,31]. Similar properties for both bilayers were found and, therefore, it was concluded that the interaction between PC and cholesterol as mentioned above did not occur or was unlikely to play an important role.

On the other hand, Zaslavsky et al. [32] reported that the addition of cholesterol increases

relative hydrophilicity of the lipid membrane surface via separation of the phospholipid head groups. This will cause an increase in the head-group hydration. If the hydrolysis rate were dependent on the hydration of the headgroup, in pure PHEPC bilayers the addition of cholesterol would accelerate the hydrolysis rate due to the enhanced hydration.

The effect of cholesterol on the hydrolysis kinetics of partially hydrogenated egg PC was investigated at different pH values at 60°C [16]. No difference was observed in the hydrolysis rate of partially hydrogenated egg PC in the liposome dispersions with and without cholesterol and concluded that the hydrolysis kinetics of partially hydrogenated egg PC were not affected by the presence of cholesterol in liposomal bilayers.

PHEPC used in the above-mentioned study was in the liquid crystalline state above 0°C and, therefore, had no transition from the fluid crystalline to gel state above 0°C [7]. Since the addition of cholesterol into gel state bilayers counteracts lipid phase transition, cholesterol incorporation into gel bilayers such as saturated soybean PC, DPPC and DSPC may bring about the disappearance of the discontinuity in the Arrhenius curve as was observed in Fig. 6.

Hydrolysis kinetics of phospholipids in charged liposomes

In pharmaceutical liposome formulations a charged phospholipid is regularly one of the bilayer-forming (phospho)lipids as it tends to improve the physical stability of liposomes by reducing the rate of aggregation and fusion [13,33]. Moreover, Talsma et al. [34] suggest that in the absence of a charged phospholipid, vesiculation is incomplete upon hydration of the cast lipid film. Addition of a charged phospholipid results in the formation of smaller liposomes and a higher encapsulation efficiency.

Incorporation of charged molecules into phospholipid bilayers generates an electrostatic charge and thereby an electrostatic surface potential which brings about a redistribution of cations and anions, including protons and hydroxyl ions, at the bilayer-water interface [35]. The Gouy-Chapman theory of the diffuse double layer

describes the relationship between surface charge density, surface potential and the concentration of the ions present in the bulk aqueous solution [35,36]. After calculating the surface potential and taking into account the interaction of cations other than protons with the charged liposome surface, the pH at the liposome surface can be calculated with the Boltzmann equation [35].

The pH at the liposome surface can also be measured experimentally with pH-sensitive probes [37]. 7-Hydroxycoumarin covalently attached to a long hydrocarbon chain is a good example to be used for this purpose. This probe can intercalate into the lipid bilayer while its fluorophore is in the lipid-water interface. Its pH-dependent excitation and emission spectrum allows determination of the pH at the bilayer-water interface. In all cases, a good agreement was obtained between the experimentally measured surface pH and the surface pH predicted by application of the Gouy-Chapman theory.

Recently, hydrolysis kinetics of partially hydrogenated egg PC and egg PG have been investigated under different conditions [23]. Different electrostatic profiles in the aqueous phase around the bilayers were obtained by varying the charged phospholipid content (egg PG) of the liposomes and the ionic strength of the bulk aqueous solution. The pH at the bilayer-water interface was calculated with the Gouy-Chapman theory. Hydrolysis kinetics of both phospholipids in buffered aqueous dispersions followed pseudo first-order reaction kinetics. Upon analysis of the data it was demonstrated that the differences between k_{obs} -values for highly charged bilayers and those for neutral bilayers can be ascribed to surface pH differences. It is the surface pH that controls phospholipid hydrolysis kinetics in liposomal bilayers and not bulk pH.

Hydrolysis rates of egg PC and egg PE were determined at pH 12.7 and at 40°C in mixed micelles containing the phospholipids separately and in a mixture of 1:1 (molar ratio) [12]. When hydrolysis rates of the pure phospholipids were determined, egg PC hydrolysed three times faster than egg PE (Table II). In a mixture of the phospholipids the difference was less pronounced. In that case egg PC hydrolysed around twice as

fast as egg PE. At pH 12.7 the PC molecule has no net charge (neutral), while PE is negatively charged due to deprotonation of the amine group (pK_a of the amine group is around 11.0) [35]. Although the authors do not give a full analysis of the possible mechanism behind this observation, it can be hypothesised that the difference and the variation of the hydrolysis rates can be ascribed to changes of the pH in the close proximity of the phospholipid molecules in the mixed micelles.

Physical stability of chemically degraded liposomes

The physical stability of liposomes concerns two parameters: (i) changes in the average particle size and size distribution due to vesicle aggregation and fusion and (ii) loss of entrapped drug due to leakage.

Changes in the average particle size and size distribution of a liposome dispersion due to aggregation and fusion are strongly affected by (phospho)lipid composition, medium composition and pH [37]. Liposomes which lack a net electrical charge tend to be less stable towards aggregation than charged liposomes. Thus, aggregation can be prevented or slowed down by incorporation of a charge-carrying lipid into the liposomal formulation. Special attention must be paid to the composition of the hydration medium: the hydration medium should not contain any polyvalent cations, which induce aggregation and, subsequently, fusion of the liposomes. If polyvalent ions are present in the hydration medium, the destabilizing effect might be overcome by the use of chelating agents such as EDTA [37].

The loss of encapsulated, water-soluble drugs due to leakage depends on the composition of the liposome, its size and the physical state of the bilayer-forming lipids (gel or liquid crystalline state) [37]. In general, leakage of drugs from gel state liposomes is slower than from liquid crystalline state liposomes [38]. However, storage around the phase transition temperature enhances the permeability of gel state bilayers [39]. Incorporation of substantial fractions of cholesterol decreases the bilayer permeability. For example a dramatic decrease in permeability of liposomes for carbox-

yfluorescein (in liquid crystalline state) by addition of 40% cholesterol has been reported by Crommelin and Van Bommel [38]. On the other hand, cholesterol incorporation into gel state liposomes, which were stored at 4°C, did not affect the permeability of the bilayer (Fig. 8).

Changes in bilayer permeability can be the consequence of chemical degradation of bilayer-forming phospholipids. The permeability for sucrose of liposomes composed of PC and PA was studied in aqueous dispersions by Hunt and Tsang [40]. Dispersions exposed to air and light showed a dramatic increase in permeability after a 10-day induction period. In α -tocopherol-containing liposome dispersions the induction period was longer. After 6 months no significant increase in the bilayer permeability was observed. These results indicate that oxidative degradation can have pronounced effects on the permeability of bilayers.

The second degradation pathway of phospholipids in aqueous liposome dispersions is hydrolysis. As already mentioned above, lysophospholipids and free fatty acids are produced as a result of phospholipid hydrolysis. These hydrolysis products have amphiphilic properties different from the 'parent' lipids and can, therefore,

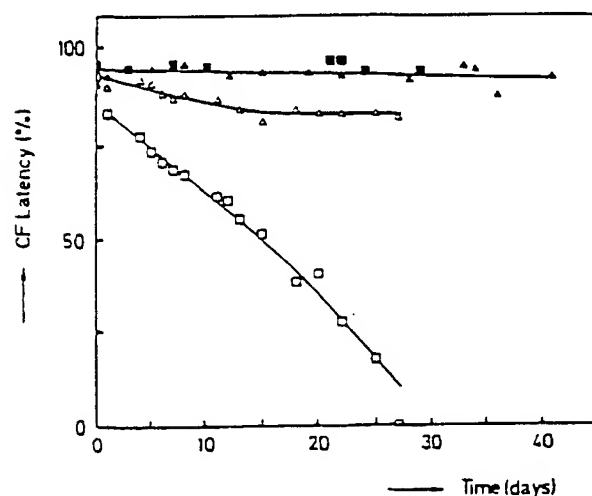


Fig. 8. CF latency in reversed evaporation vesicles (REV) dispersed in iso-osmotic sodium chloride/0.01 M Tris solutions, pH 7.4, stored at 4–6°C. Data from Ref. 38. □, PC/PS (9/1, molar ratio); △, PC/PS/cholesterol (10/1/4, molar ratio); ■, DPPC/DPPG (10/1, molar ratio); ▲, DPPC/DPPG/cholesterol (10/1/5, molar ratio)

affect the bilayer properties. For example, long fatty acyl chain-bearing lysophospholipids do not adopt a bilayer configuration, but organize themselves in a micellar structure when dispersed in water [41]. Bilayer structures are destabilized when they contain an elevated concentration of lysophospholipids. As a consequence of destabilization, the bilayers become more sensitive to fusion and can undergo morphological changes affecting their permeability [42–45]. Enhanced leakage of glucose from DMPC liposomes was shown in a study where the effect of exogenous LPC on the physical stability of liposomes was investigated [46]. Similar results were also reported for exogenous LPC containing egg PC liposomes where carboxyfluorescein was used as a hydrophilic marker molecule [47]. In the above-mentioned studies leak-out rate and/or leak-out versus time profiles of the encapsulated hydrophilic marker molecules were used as parameters to describe the bilayer permeability.

Recently, the leak-in rate of calcein was determined in PHEPC/EPG-containing liposomes with and without cholesterol [48]. Calcein was chosen as a hydrophilic marker molecule because its efflux from liposomes was insensitive to pH changes of the hydration medium in the pH range 5.5–8.0 [49]. The leak-in rate of calcein into liposomes is enhanced when increasing concentrations of exogenous LPC are incorporated into bilayers at 40°C in liposomes without cholesterol (Fig. 9). Cholesterol addition to these liposomes reduced the leak-in rate of calcein.

The second primary hydrolysis products of phospholipids are free fatty acids. Incorporation of fatty acids into liposomal bilayers increases the tendency of liposomes to aggregate [50]. The effect of exogenous myristic acid on the permeability of DMPC liposomes was investigated at 22°C (below the transition temperature of DMPC) and 38°C (above the transition temperature of DMPC). Bilayer permeability to K^+ was not affected up to a concentration of 5% myristic acid (on a molar basis). Above that critical concentration of exogenous myristic acid, a dramatic decrease in permeability is observed with liquid crystalline state bilayers (38°C). This effect is less pronounced in gel state bilayers (Fig. 10) [51].

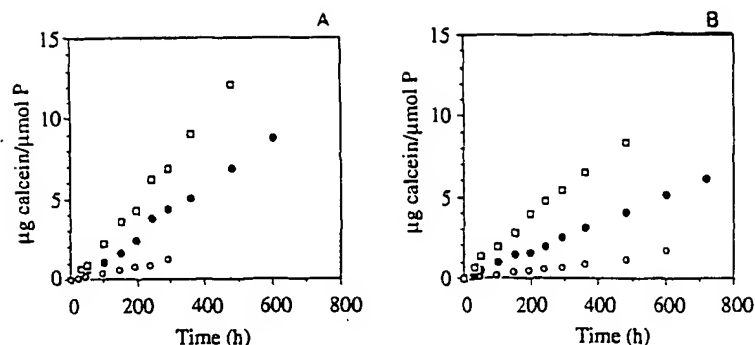


Fig. 9. Leakage of calcein into exogenous LPC-containing liposomes at 40°C. Data from Ref. 48. A, PHEPC/EPC (10/1, molar ratio) liposomes; O, fresh; ●, 5% LPC added; □, 20% LPC added; B, PHEPC/EPC/cholesterol (10/1/4, molar ratio) liposomes; O, fresh; ●, 5% LPC added; □, 20% LPC added.

In reality, hydrolytic degradation products are not added artificially to the bilayer upon storage, but they are formed during storage and gradually influence bilayer characteristics. At least in the initial stage of the process, the hydrolysis products are part of the bilayers. Therefore, in order to gain more insight into the effect of hydrolysis products on bilayer permeability, experiments were performed with liposome dispersions containing free fatty acids and lysophospholipids formed in-situ. In these experiments liposome dispersions were stored over different time intervals at elevated temperatures to obtain partially hydrolysed phospholipids with different levels of degradation. Afterwards, the leak-in rate of the non-bilayer-

interacting hydrophilic marker molecule calcein was determined at 40°C. Exogenous LPC-containing liposomes were taken as the control. The leak-in rate of calcein was enhanced by the increasing concentrations of exogenous LPC. On the other hand, in liposomes containing partially hydrolysed phospholipids, a drop in leak-in rate was found up to 10% hydrolysis (corresponds to around 10% LPC) of the phospholipids involved. Above that degree of hydrolysis the leak-in rate started to increase as in exogenous LPC-containing liposomes (Fig. 11). Both liposome dispersions with and without cholesterol followed a similar pattern [48]. The permeability enhancement effect of LPC was assigned to the tendency of LPC molecules to interdigitate in phospholipid bilayers. Apparently, up to a certain level the

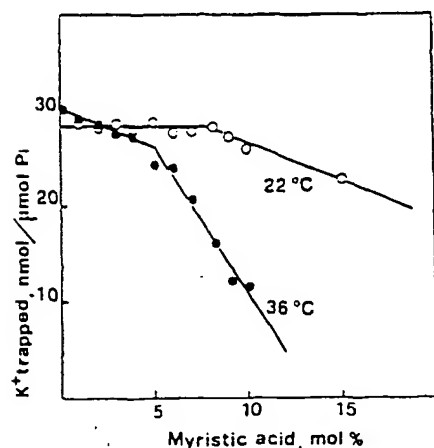


Fig. 10. K^+ trapped into the vesicles. Dependence on the amount of exogenous myristic acid. Data from Ref. 51; O, 22°C and ●, 36°C.

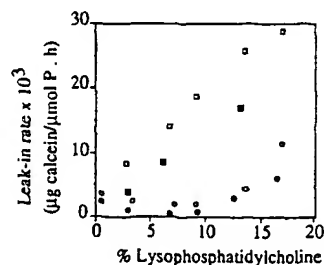


Fig. 11. Leak-in rate of calcein through liposomal bilayers at 40°C as a function of LPC content of liposomes. Data from Ref. 48. O, PHEPC/EPC (10/1, molar ratio) hydrolysed ('aged') liposomes; ●, PHEPC/EPC/cholesterol (10/1/4, molar ratio) hydrolysed ('aged') liposomes; □, PHEPC/EPC (10/1, molar ratio) LPC added liposomes; ■, PHEPC/EPC/cholesterol (10/1/4, molar ratio) LPC added liposomes.

presence of both LPC and free fatty acids has a stabilizing effect on the bilayer permeability. In the initial stage the free fatty acids effectively neutralise the LPC-destabilizing effect and the presence of both degradation products makes the bilayer even less permeable than with phospholipids alone.

Quality control of liposome dispersions

Quality control has always been a key issue in pharmaceutical production. It starts with the qual-

ity control of raw materials to be used for the production, is followed by in process control in order to find out batch to batch variations and/or any problem which can affect the quality of the end product and it ends with the quality control of the final product. This sequence of validated procedures has to be followed for each production batch so that regulatory authorities can be assured that reproducible pharmaceuticals are being produced. Although no guidelines have been published by regulatory authorities for the quality control of liposome-based pharmaceuticals, a concept for

TABLE III_a

Quality control assays of pharmaceutical liposomal formulations.

	Methodology
<i>Characterization assays</i>	
pH	pH meter
Osmolarity	Osmometer
Phospholipid concentration	Lipid phosphorus content (Fiske and Subbarow or Bartlett method)
Cholesterol concentration	Cholesterol oxidase assay, HPLC
Drug concentration	Spectrophotometry, HPLC or other chromatographic procedure
<i>Biochemical stability assays</i>	
pH	pH meter
Phospholipid peroxidation	Conjugated diens, lipid peroxides, thiobarbituric acid (TBA) test and fatty acid composition (GC)
Phospholipid hydrolysis	TLC, HPLC
Cholesterol autooxidation	TLC, HPLC
Antioxidant degradation	TLC, HPLC
Drug degradation	TLC, HPLC, Spectroscopy
<i>Physical stability assays</i>	
Vesicle size distribution:	
submicrometer range	Photon correlation spectroscopy, Gel exclusion chromatography and specific turbidity
micrometer range	Coulter counter, Laser diffraction and light microscopy
Electrical surface potential and surface pH	Zeta potential measurements, use of electrical field or pH sensitive probes
Percentage of free drug	Gel exclusion chromatography, ion exchange chromatography and protamine precipitation
Dilution dependent drug release assay	Dilution effect (0–10 000-fold) on liposomal drug/PL ratio
Drug/phospholipid ratio	Determination of drug and phospholipid content
<i>Biological assays</i>	
Sterility	Aerobic and anaerobic bottle culture
Pyrogenicity	Rabbit and/or Limulus amoebocyte lysate (LAL) tests
Animal toxicity	Monitor survival
Relevant body fluid-induced leakage	Gel exclusion chromatography, ion exchange chromatography and protamine precipitation

^aTaken from Ref. 52

the characterisation and quality control of liposome-based pharmaceuticals has been published by Barenholz and Crommelin (Table III) [52]. As presented in Table III, quality control procedures for a pharmaceutical liposome dispersion should deal with three different degrees of validated assays: chemical characterisation and stability assays, physical characterisation and stability assays and biological assays.

Chemical characterisation and stability assays describe the phospholipid composition of fresh and stored liposome dispersions. They are discussed in this article. Other quality control assays such as the determination of the average particle size and size distribution and determination of free and encapsulated drug concentrations are dealt with in other chapters.

As already mentioned in the second part of this article, assessment of the chemical stability of phospholipids on storage in terms of phospholipid hydrolysis cannot be based on the determination of lysophospholipids present in the liposome dispersions. As lysophospholipids are intermediate hydrolysis products, their concentration can reach a steady-state level after a period of time while phospholipid hydrolysis still takes place. Therefore, in order to gain full insight into the phospholipid hydrolysis process, the concentrations of parent phospholipids, lysophospholipids, fatty acids and glycerophospho compounds must be determined.

Colorimetric methods as described by Fiske and Subbarow [53] and Bartlett [54] are widely used for the determination of the total phospholipid content of liposomes e.g. to minimise batch-to-batch variation. In order to determine the concentration of individual phospholipids, analytical methods which are able to separate phospholipids present in a liposome formulation must be used. Most widely used methods are thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Improved sensitivity, high precision and efficient separation and quantitation of minor components present in the samples with larger sample loadings without loss of resolution are the advantages of HPLC over TLC. Therefore, in our opinion HPLC is the method of choice in phospholipid analysis. Among many

other methods described in the literature and reviewed recently [55], an example of a validated HPLC method is given below. This procedure has been used in studies where the hydrolysis kinetics of phospholipids in aqueous liposome dispersions have been described.

Separation of PC, PG and their hydrolysis products lysophosphatidylcholine and lysophosphatidylglycerol was achieved on an amino phase column (Zorbax-NH₂, 25 cm × 4.6 mm i.d.) with a mobile phase consisting of acetonitrile/methanol/10 mM ammonium dihydrogen phosphate solution pH 4.8 (64/28/8, v/v) which was delivered at a flow rate of 1.5 ml/min [56]. A chromatogram obtained after the analysis of a mixture of phospholipids (DMPC, DMPG, MPC and MPG),

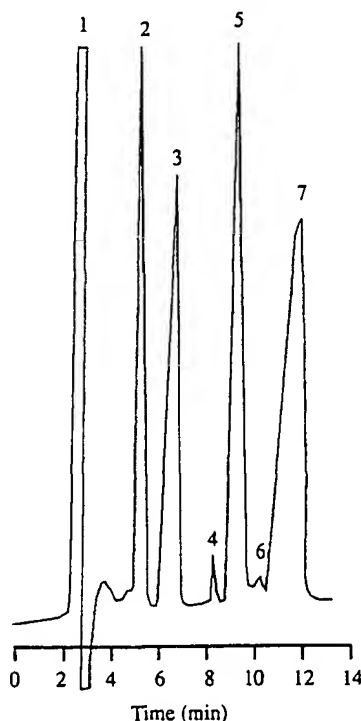


Fig. 12. HPLC chromatogram of phospholipids. Data from Ref. 48. HPLC conditions: mobile phase, acetonitrile/methanol/10 mM ammonium dihydrogen phosphate solution, pH 4.8, (64/28/8, v/v); flow rate, 1.5 ml/min; detection, RI. (1) Solvent front; (2) dimyristoylphosphatidylcholine (DMPC); (3) dimyristoylphosphatidylglycerol (DMPG); (4) 2-myristoyl lysophosphatidylcholine (MPC); (5) 1-myristoyl lysophosphatidylcholine (MPC); (6) 2-myristoyl lysophosphatidylglycerol (MPG); (7) 1-myristoyl lysophosphatidylglycerol (MPG).

is presented in Fig. 12. Under these conditions separation of the isomers of lysosphospholipids was also obtained (Fig. 12). Furthermore, separation of phospholipid classes other than those given above has been achieved as well. Calibration curves obtained were linear over two orders of magnitude and detection limits of PC, PG, LPC and LPG were 22, 29, 30 and 50 $\mu\text{g/ml}$ with an injection volume of 20 μl . Method precision for a standard phospholipid mixture and for a PC-PC-containing liposome dispersion was in the range 0.6–4.5% (relative standard deviation).

Different detectors have been described to be used for phospholipid detection after column separation. These detectors are based on UV-VIS electromagnetic radiation absorption in the range 195–215 nm, differential refractometry (RI detector), light scattering and flame ionization. Despite the shortcomings mentioned below, UV absorption in the range 195–215 nm is regularly used at present for quantitative analysis of phospholipids. Its sensitivity varies with the degree of unsaturation of the phospholipid's fatty acyl chains: highly unsaturated phospholipids yield a good response, the response for the unsaturated phospholipids is poor. Comparison of short wavelength UV detection and RI detection has been done recently to test the potential of both detectors in phospholipid analysis. Molar responses (peak area measurements) of a series of phosphatidylcholines varying in degree of unsaturation have been determined using both detectors [56]. While the molar response of the RI detector was constant for all phosphatidylcholines tested, the molar response of the short wavelength UV detector increased proportionally to the degree of unsaturation of the fatty acid chains of the phosphatidylcholines (Fig. 13). Besides the advantage of RI detector, its incompatibility with any form of gradient elution (solvent and flow gradients) is a disadvantage when operating a method using gradient elution. Moreover, RI detector must be operated at constant temperature. Temperature changes at close proximity of the detector will cause instability of the baseline. Therefore, adjustment of the column and mobile phase temperature is recommended when the difference between outside and operation temperature is large. In general, the sensitivity of

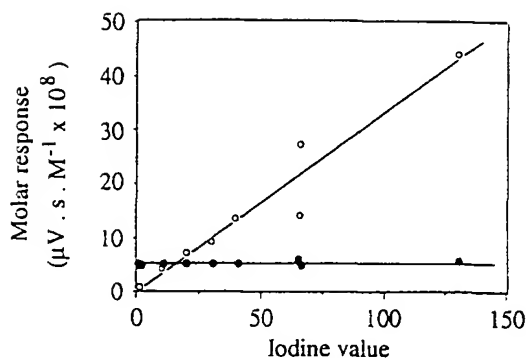


Fig. 13. The relationship between the molar response of different species of phosphatidylcholine with varying degrees of saturation. Data from Ref. 56. (●), RI; (○), UV detector set at 206 nm.

the RI detector was in the same range as the sensitivity of the UV detector when dealing with phospholipids with low degrees of unsaturation. But, the UV detector was more sensitive for the analysis of unsaturated phospholipids. As detector sensitivity of impurities such as lyso phospholipids with low degrees of acyl chain saturation is still relatively low, a new generation of more sensitive detectors with constant molar response should improve and simplify quality control procedures. Moreover, Zorbax-NH₂ columns with lower batch-to-batch variability in performance and being able to not only separate major (lyso)phospholipids, but also fatty acids in one run would contribute to simplification of the assessment of product quality in different stages of preparation of liposome dispersions.

Conclusions

Two decomposition pathways of liposomal phospholipids, oxidation and hydrolysis, have been dealt within the first part of this article. Oxidative degradation can be reduced by the use of high quality raw materials, by addition of antioxidants and by preparation of liposomes under an oxygen-free atmosphere. Storage at low temperatures reduces the rate of oxidation as well. Furthermore, the use of partially saturated phospholipids could be a better choice than the phospholipids carrying polyunsaturated fatty acyl chains (natural egg phosphatidylcholine).

In contrast to oxidative degradation, hydrolysis of phospholipids can only be fully prohibited by removal of water by means of (freeze)-drying. However, because of the physical stability problems encountered with freeze-drying of liposomes containing hydrophilic, non-bilayer interacting, low molecular weight drugs (e.g. loss of drug after rehydration and the tendency to growth of the average particle size) storage of the liposomes as an aqueous dispersion may be the preferred choice. In an aqueous dispersion, the storage temperature and pH were found to be two major parameters affecting phospholipid hydrolysis. Therefore, for long-term stability, storage at low temperatures (4–6°C, in a refrigerator) and adjustment of the pH of the dispersions to pH values close to neutral — where phospholipids have their maximum stability (pH 6.5) — is recommended. Moreover, the lowest possible buffer concentrations that ensure constant pH should be chosen as buffer species tend to accelerate the hydrolysis process.

Charged phospholipids are, in general, added to obtain a physically stable liposome dispersion. In the presence of charge a deviation of the pH at the bilayer-water interface from the bulk pH must be taken into account. The difference between the surface pH and the bulk pH can be large, especially with a high content of charged phospholipid in the liposomal bilayer and a low ionic strength in the aqueous bulk solution. On the other hand, such pH difference can be advantageous when formulating liposomes containing drugs with a maximum stability at a slightly alkaline pH. The text above indicates that with a careful formulation of the liposomes the chemical stability of liposomes can be maximised through rational conditions so that pharmaceutically acceptable shelf-lives for aqueous liposomal dispersions may be achieved.

Studies in the seventies showed that the presence of exogenous lysophospholipids increases the permeability of liposomal bilayers [45,46]. In contrast to that finding, we found that the permeability of bilayers was not negatively affected by the presence of hydrolysis products (both lysophospholipids and fatty acids) formed in situ up to around 10% phospholipid hydrolysis. The implication of this state of partial hydrolysis on liposome

behavior in vivo has not been investigated yet. Both their disposition and safety may be affected by the presence of LPC and fatty acid constituents which are known to have a high affinity to other blood constituents. Therefore, additional in vivo studies should be performed using 'aged' liposome dispersions containing in situ-formed hydrolysis products in order to provide better strategies for the assessment of shelf-life of liposome-based pharmaceuticals.

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Phospholipid-Stabilized Nanoparticles of Cyclosporine A by Rapid Expansion from Supercritical to Aqueous Solution

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ABSTRACT

The purpose of this research was to form stable suspensions of submicron particles of cyclosporine A, a water-insoluble drug, by rapid expansion from supercritical to aqueous solution (RESAS). A solution of cyclosporine A in CO₂ was expanded into an aqueous solution containing phospholipid vesicles mixed with nonionic surfactants to provide stabilization against particle growth resulting from collisions in the expanding jet. The products were evaluated by measuring drug loading with high performance liquid chromatography (HPLC), particle sizing by dynamic light scattering (DLS), and particle morphology by transmission electron microscopy (TEM) and x-ray diffraction. The ability of the surfactant molecules to orient at the surface of the particles and provide steric stabilization could be manipulated by changing process variables including temperature and suspension concentration. Suspensions with high payloads (up to 54 mg/mL) could be achieved with a mean diameter of 500 nm and particle size distribution ranging from 40 to 920 nm. This size range is several hundred nanometers smaller than that produced by RESAS for particles stabilized by Tween 80 alone. The high drug payloads (~10 times greater than the equilibrium solubility), the small particle sizes, and the long-term stability make this process attractive for development.

KEYWORDS: supercritical fluid, carbon dioxide, rapid expansion, water-insoluble

INTRODUCTION

The often low bioavailability of water-insoluble drugs leads to poor pharmacokinetic performance in the body. Techniques to improve bioavailability in oral or parenteral applications of water-insoluble drugs include powder micronization, the formation of micron- and submicron-size dispersions, solubility enhancement in aqueous solution by addition of appropriate surfactants, organic solvents or buffers, or drug-carrier systems such as liposomes. The payloads for drugs in liposomes are often limited because of the low volume fraction of hydrophobic regions.¹ The use of surfactants or organic solvents in parenteral administration can lead to phlebitis, anaphylaxis, hypotension, or even vasodilation.¹ Traditional micronization techniques such as spray-drying,^{2,3} emulsion-solvent extraction,⁴⁻⁶ and processes based on high shear (eg, microfluidization, high-pressure homogenization, ball milling, air jet milling)⁷⁻¹¹ can have certain drawbacks. With many of these techniques, particle size distributions tend to be broad, products can be denatured by exposure to high temperatures or organic solvents, residual solvent concentrations can be high without lengthy periods for additional extraction/evaporation, or undesirable processing agents need to be separated from the final products. Yields can be well below 100% due to losses during solids handling in milling and spray drying. Milling techniques also require cumbersome solids handling. Hence, processing techniques that do not rely on organic solvents or high temperatures and that can provide small particles with narrow distributions are highly desirable.

Dissolution rates of poorly-water soluble drugs may be increased by reducing the particle size to increase the surface area and by inhibiting crystallization to form amorphous particles. Both of these factors may be achieved by phase separation techniques that include rapid nucleation rates and prevention of particle growth. The process rapid expansion from supercritical solution (RESS) may be used to accomplish these goals according to theoretical models of nucleation. The expansion from the supercritical state to atmospheric pressure reduces the solvent density (or strength) and initiates intense nucleation.¹²⁻²² The particle formation steps

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include nucleation, condensation of solute molecules about the nuclei, and coagulation of particles in the free jet expansion. Recent work by Charoenchaitrakool et al has produced 2.5- μm particles by RESS with enhanced dissolution rates for the poorly water-soluble compound ibuprofen, likely due to the reduction in both particle size and crystallinity.²³ If coagulation can be minimized, it should be possible to produce 20- to 50-nm drug particles.²⁴ Similar findings have been noted by M. Weber and M. Thies (letter, March, 2001).

A novel process, rapid expansion from supercritical to aqueous solution (RESAS), has been developed to reduce the coagulation rate in the free jet expansion of RESS.²⁵ The supercritical solution is expanded through an orifice or tapered nozzle into an aqueous solution containing a stabilizer to minimize particle aggregation during free jet expansion. Previously, Young et al demonstrated the ability for Tween 80, a nonionic polysorbitan ester, to stabilize 400 to 700 nm cyclosporine particles produced by RESAS.²⁵ CO₂ was chosen as the supercritical fluid of interest as it is an environmentally benign solvent that is nonflammable, inexpensive, and essentially nontoxic. It also has relatively mild critical conditions, critical temperature (T_c) = 31°C, critical pressure (P_c) = 73.8 bar, and so allows processing at moderate temperatures to prevent thermal degradation. Sun et al have demonstrated a technique to form PbS nanoparticles by expanding a supercritical fluid containing one reactant, Pb(NO₃)₂, into a liquid solution containing a second reactant, Na₂S.²⁶

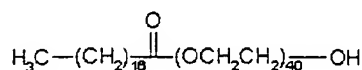
In this study, the emphasis is on phospholipids to minimize coagulation in RESAS. Phospholipids are amphiphilic molecules, usually consisting of 2 lipophilic tails, which, when added to water, rapidly aggregate and form liposomes. Depending on the chemical compositions of the phospholipids, the concentration, and the method of formation, a variety of sizes and structures can be formed, such as 1- to 10- μm multilamellar vesicles (MLVs), 0.1- to 1.0- μm large unilamellar vesicles (LUVs), and $<0.1\text{-}\mu\text{m}$ small unilamellar vesicles (SUVs).^{27,28} In this study, the focus is on SUVs. High drug:lipid ratios with particle sizes $<1.0\text{ }\mu\text{m}$ have been achieved by micronizing drugs while in the presence of phospholipid stabilizers.¹ It is suggested that in these systems the drug partitions into the bilayers of the SUVs employed, and colloidal drug particles are stabilized by a monolayer of phospholipid and either successive bilayers or small loosely associated vesicles.

The goal of this study was to use phospholipid vesicle solutions to stabilize nanoparticle aqueous dispersions of cyclosporine, a water-insoluble immunosuppressant, with substantial payloads (eg, above 20 mg/mL) by RESAS. To place these experiments in perspective, new RESAS results are presented for stabilization by a series of micelle-forming surfactants (without phospholipids). The phospholipids were

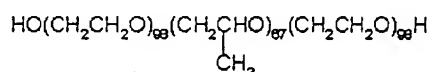
mixed with small amounts of micelle-forming surfactants to enhance fluidity of the surfactant bilayers. The results were compared for aqueous solutions stabilized with vesicles with only micelle-forming surfactants to provide mechanistic insight into the stabilization processes. The effects of several variables, such as drug concentration in the suspension, stabilizing solution temperature, preheater temperature, and flow rate, were examined.

MATERIALS AND METHODS

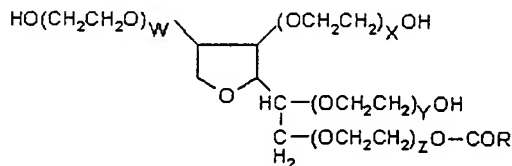
Cyclosporine was obtained from North China Pharmaceutical Corporation (Shijiazhuang, China) and used without any further purification. Lipoid E80 (Vernon Walden, Madison, NJ), Phospholipon 100H (American Lecithin, Oxford, CT), Myrj 52 (ICI Americas, Wilmington, DE), Pluronic F127 (BASF, Ludwigshafen, Germany), methanol (high performance liquid chromatography [HPLC] grade, EM Science, Gibbstown, NJ), and Tween 80 (Aldrich, Milwaukee, WI) were used without further purification. Mannitol, sodium lauryl sulfate, and sodium deoxycholate were purchased from Sigma (St Louis, MO) and used without further purification. Water was purified to Type I reagent grade by passing it through a Barnstead (NANOpure II) filtration system (Barnstead International, Dubuque, IA). Instrument grade CO₂ (Matheson, Albuquerque, NM) was used for all of the experiments. The structures of the various surfactants used in this study are shown in Figure 1.



(A) Myrj 52, 2032 g/mol



(8) Pluronic F127, ~12 000 g/mol



$$W+X+Y+Z=20$$

$$R = \text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7-$$

(C) Tween 80, ~1160 g/mol

Figure 1. Structures of the surfactants used in this study: (A) Myrj 52, 2032 g/mol, (B) Pluronic F127, ~12 000 g/mol, and (C) Tween 80, ~1160 g/mol.

HPLC combined with UV detection was used to quantify the drug concentration from the aqueous suspensions. The column used for HPLC was a 250-mm long C-18 column (SGE ODS, 5 μ m). Samples were prepared by withdrawing 0.5 mL of the suspension and adding to 5 mL of methanol, of which 9.6 μ L was actually injected onto the column. The mobile phase consisted of pure methanol, and the detection wavelength was 210 nm.

The solubility of cyclosporine in the surfactant solutions was determined from a saturated solution at 25°C. Excess drug was added to 10 mL of each surfactant solution and allowed to equilibrate with stirring for 1 week at 25°C. The dissolved drug content was determined by the above-mentioned HPLC method by analyzing a filtrate of each saturated solution.

The intensity-weighted particle size distribution was determined by Dynamic Light Scattering (DLS) via a Brookhaven Zetaplus (Brookhaven Instruments, Holtsville, NY). Particle size measurements were made within 24 hours of preparation and at 1-month time intervals. Multimodal size distributions were determined by a nonnegative least squares method, and the mean diameters and size ranges were reported.

X-ray diffraction data was taken with a PW1720 x-ray generator (Philips Electronic Instruments, Inc, Mahwah, NJ). Suspensions examined were frozen and lyophilized, and the dry powder remaining was examined. Samples containing liquid surfactants, such as Tween 80, could not be examined by this technique, only those forming a dry powder.

Differential scanning calorimetry (DSC) was used to determine the phase transitions of the phospholipids. A TA Instruments (New Castle, DE) DSC 2920 calorimeter was used. Typically, 8 to 15 mg of sample was loaded into the pan. A heating rate of 1°C/min was used to scan over the temperature range desired.

Confirmation of vesicle and particle size and structure was determined by transmission electron microscopy using a Philips EM208 microscope (Philips). Diluted samples were dropped onto copper grids. The grid was allowed to stand for 5 minutes before the negative stain was dropped onto the grid. The stain used was a 1.0% (wt/wt) solution of uranyl acetate, which had been brought to a neutral pH to prevent damage to vesicle structure.²⁹ After staining for 5 minutes, the grid was washed lightly (3 drops) with pure water and then the excess water was removed by touching the edge of the grid to an absorbent cloth.

Preparation of Phospholipid Vesicles

Phospholipid solution "A" was typically made with an overall aqueous batch size of 200 g. First, Tween 80 and mannitol

were added to the requisite amount of water and stirred until completely dissolved. The solution was chilled in an ice water bath, and then the phospholipid was added. The solution was stirred and sonicated (Branson Sonifier 250, Branson Ultrasonics Corporation, Danbury, CT) while still chilled to break up any large clumps. At this time, the pH was adjusted to 7.0 by adding 0.1M NaOH. This solution was then passed 30 times through a high-pressure homogenizer (Avestin Emulsiflex C-5, Avestin Inc, Ottawa, Ontario, Canada) at a shear pressure drop of 15 000 psig to produce small unilamellar vesicles. The outlet flow from the pump was passed through a chilling tube, which was submerged in an ice bath, before returning to the pump feed supply to keep the temperature of the phospholipid solution entering the pump at 10°C. At the end of the run, the pH was again checked and, if necessary, 0.1M NaOH was added to bring the final pH to between 7.5 and 8.0. This solution was then refrigerated at 4°C until ready for use.

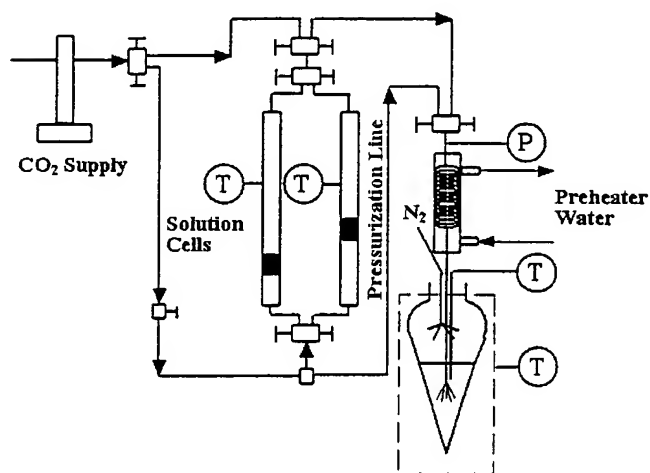


Figure 2. Schematic of apparatus used for rapid expansion from supercritical to aqueous solution (RESAS). T: temperature indicator, P: pressure indicator.

Rapid Expansion from Supercritical to Aqueous Solution

The RESAS apparatus is shown in Figure 2. The CO₂ was supplied to the system by means of a high-pressure computer-controlled syringe pump (ISCO). The 2 solution cells were composed of 4-foot-long, 11/16-inch inner diameter (id) \times 1-inch outer diameter (od), stainless steel tubes set up in parallel and equipped with pistons. Each cell was loaded with a predetermined mass of drug above the piston, sealed, and then loaded with a known volume of CO₂. The back side (or bottom) of the piston was subsequently pressurized by CO₂ to the desired pressure. Each cell was heated by four

2-foot-long strips of heating tape connected to heating controllers to maintain the temperature to within $\pm 0.3^{\circ}\text{C}$. For initial solution equilibration and mixing, only 2 of the heating tape strips were heated (the second and fourth from the top) to produce density fluctuations within the fluid to aid mixing. At the same time, the pressure within the cell was cycled via the syringe pump to create further mixing by moving the piston up and down. Typically, the solution would come to the equilibrium concentration within 24 hours. The solution concentration was checked by spraying the solution into pure methanol to collect dissolved drug, and the sample was analyzed by HPLC. If the aforementioned mixing technique was not utilized it could take the solution up to a week to reach equilibrium. The preheater consisted of a 30-foot-long piece of 1/16-inch od \times 0.03-inch id stainless steel tubing coiled within a 1½-inch diameter \times 20-foot-long tube (column heater, Alltech Associates, Deerfield, IL) with heated water circulating through it at a rate of 2.4 L/min. This heat exchanger allowed very uniform heating of the preheater coil, preventing hot spots, which could otherwise produce crystallization of the drug within the coil.

For most of the experiments, the nozzle was made from a 10-inch-long, 1/16-inch od \times 0.03-inch id stainless steel tube, in a similar manner to that previously described.²⁵ Two sizes of nozzle were made by filing the end of the nozzle, allowing flow rates of 0.88 mL/min or 2.5 mL/min at a constant pressure drop of 345 bar. Figure 3 provides an example of what the spray pattern looks like exiting the 2.5-mL/min nozzle operated at a pressure drop of 345 bar. For comparison purposes, a 10-inch-long piece of 50- μm id fused silica capillary tubing (Polymicro Technologies, Phoenix, AZ) was used as the nozzle for a few experiments.

The aqueous stabilizing solution was held in a 125-mL separatory funnel. The expanding diameter of the funnel helps to destabilize foam formation created by CO_2 bubbling through the solution, while providing sufficient depth for small volumes of liquid to allow the nozzle to be submerged. The nozzle was submerged approximately 1 cm below the surface of the aqueous solution. In this way, the particles being formed are brought into close contact with the stabilizing solution. The separatory funnel was submerged within a temperature-controlled water bath. A thermocouple was submerged next to the nozzle in the stabilizing solution to measure the local temperature of the solution. To suppress and drain any foam produced during RESAS, N_2 gas was blown down on top of the foam at 7.8 L/min and 2 bar into the funnel approximately 4 cm above the foam through four 1/16-inch od \times 0.03-inch id stainless steel tubes. Prior to each experiment, 10 mL of the stabilizing solution was filtered with a 0.2 μm sterile disposable syringe filter (Whatman, Kent, UK) and then added to the funnel. Imme-

diately prior to collecting the particles, the stabilizing solution in the funnel was submerged in the heated water bath to bring it to the desired collection temperature.

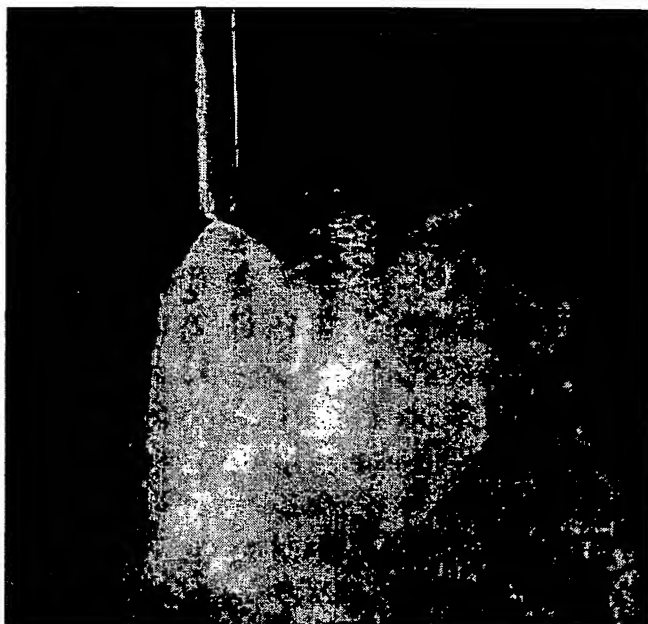


Figure 3. Spray profile for a CO_2 solution expanding through a tapered elliptical nozzle with a flow rate of 2.5 mL/min at 345 bar.

To produce the suspensions, first the preheater and nozzle assemblies were prepressurized with CO_2 . The prepressurization prevented plugging of the nozzle. Once the flow of CO_2 had equilibrated, the flow was switched so as to push the solution out of the cell and through the nozzle. Initially, the spray was conducted by spraying into a separatory funnel containing pure water. Once drug particles began to accumulate in the water, the separatory funnel was switched to one of the prepared and preheated stabilizing solutions. A measured volume (as measured by the syringe pump) of solution was sprayed to produce a suspension of a desired concentration. Upon completing the spray, the stabilizing solution was replaced by pure water again, and the flow switched back to pure CO_2 . Sufficient CO_2 was allowed to spray through the nozzle to ensure that all drug was swept out of the system to prevent nozzle plugging upon depressurization. For all of the samples using phospholipid-based surfactants, the pH of the suspension was adjusted again, after the spray was completed, to 7.0 by adding sufficient 0.1M NaOH. Typically the pH of the phospholipid solution went from 7.5 to 3.5 during the course of the spray. pH neutralization was required to ensure long-term stability of the phospholipids. The samples were stored with a N_2 headspace by purging the air from the sample vial through a sep-

Table 1. Solubility of Cyclosporine in Various Surfactant Systems at 25°C*

Surfactant Name	Surf Concentration in H ₂ O % (g/g)	Drug Solubility (mg/mL)	Drug/Surf ratio (g/g)
Tween 80 [†]	1	0.56	0.056
	2	1.2	0.060
Myrj 52	2	1.7	0.085
SLS	2	27.0	1.4
Pluronic F127	2	0.01	0.0005
Lipoid E80/Tween 80/mannitol	10/2/5.5	4.75	0.04
	1/0.2/0.55	0.32	0.03

*SLS indicates sodium lauryl sulfate; and surf, surfactant.

[†]Reference 26.

tum in the vial cap to prevent oxidation of the phospholipid components. The samples were refrigerated at 4°C.

RESULTS AND DISCUSSION

Cyclosporine Solubility

The phase behavior of cyclosporine in CO₂ has previously been reported.²⁵ At 30°C and 345 bar, 1.0% (wt/wt) of cyclosporine is readily soluble in CO₂. All of the RESAS experiments in this work were performed with an initial solution concentration of 1.0% (wt/wt). It is important to note that the solubility of cyclosporine in CO₂ decreases with temperature at constant pressure (cloud point increases). The solubilities of cyclosporine in the surfactant solutions used for stabilization were measured to serve as a control, as shown in Table 1 at 25°C. The goal of RESAS was to produce suspensions with a drug/surfactant ratio much higher, and at least twice as high, as is obtained from the equilibrium solubility in the surfactant solution. As a result, sodium lauryl sulfate (SLS) was not chosen as one of the surfactants to examine since the solubility of cyclosporine in this solution is quite high.

Effect of Surfactant Type on Particle Size

While the bulk of the RESAS experiments will focus on phospholipid-based systems, micelle-forming surfactants including Tween 80, Pluronic F127, and Myrj 52 were studied for comparison, as shown in Table 2. All of the experiments shown in Table 2 had the following initial conditions: a drug concentration of 1.0% (wt/wt) in CO₂, a solution temperature of 30°C, a preheater temperature of 60°C, a pressure drop of 345 bar with a nozzle that produced a flow rate of 2.5 mL/min, and a stabilizing solution bath temperature of 25°C. The table shows the final concentration of drug in the suspension as measured by HPLC, the mean particle size, the particle size distribution (with the relative percentage of particles found in each peak for multiple peak

distributions), and the drug to surfactant ratio for the equilibrium solubility as well as for the actual suspension.

As seen in Table 2, Tween 80 stabilized particles with mean diameters from 660 to 970 nm with fairly broad distributions at relatively high drug/surfactant ratios of nearly 0.6, which was 10 times the equilibrium solubility. These results agree reasonably well with previous work.²⁵ The new experiments utilized smaller aqueous stabilizer volumes and higher flow rates that did not seem to modify the particle size. To minimize aggregation resulting from particle collisions, the surfactant must reach the surface of the primary particle rapidly and orient such that it can provide steric stabilization. Previously,²⁵ the particle size increased markedly for a drug:surfactant ratio >0.6 due to insufficient surface coverage. In contrast, using Pluronic F127 led to substantially larger particles despite the lower drug/surfactant ratio of approximately 0.25. It appears that this surfactant has a lower affinity for the particle surface, which is consistent with the low equilibrium solubility. The lipophilic propylene oxide group makes up only 30% (wt/wt) of the surfactant molecule, which might not be enough for sufficient adsorption at the particle surface. The use of Myrj 52 also yielded larger cyclosporine particles than Tween 80. While this surfactant has a modestly different lipophilic group than Tween 80 (stearate compared with oleate), it also has a more linear, or less bulky, hydrophilic group. Tween 80 has several ethylene oxide side chains providing greater steric repulsion in the continuous aqueous phase.

The compositions of several phospholipid-based surfactant mixtures are provided in Table 3. The 2 phospholipids included commercially available compounds Lipoid E80 (from chicken egg white) and Phospholipon 100H (hydrogenated soybean), which both consist primarily of dipalmitoylphosphatidylcholine (DPPC) and include small amounts of impurities such as phosphatidylethanolamine, sphingomyelin, and lyso-phosphatidylcholine. Each of these systems was prepared so as to have initial structures consisting of small unilamellar vesicles <1.0 µm in water. Preparations

Table 2. Effect of Surfactant Type on Cyclosporine Microparticles Prepared by RESAS of a 1.0% (wt/wt) Solution into 10.0 mL of 2.0% (wt/wt) Aqueous Surfactant Solution*

Surfactant Type	Drug Conc (mg/mL)	Yield % (wt/wt)	Particle Mean (nm)	Particle Size Distribution (nm)	Drug/Surf Ratio @ max sol	Drug/Surf Ratio (g/g)
Tween 80	6.1	68	970	210-320 (21%), 500-760 (59%), 2200-3400 (20%)	0.056	0.61
Tween 80	5.4	64	660	220-290 (25%), 700-940 (75%)		0.54
F127	4.0	46	1010	140-250 (12%), 400-860 (75%), 3400-5400 (13%)	0.0005	0.20
F127	4.7	48	1200	180-310 (7%), 520-870 (66%), 2100-4100 (27%)		0.24
Myrj 52	4.6	49	840	90-120 (2%), 310-590 (58%), 1100-2100 (40%)	0.085	0.23
Myrj 52	3.2	38	1240	150-270 (9%), 410-850 (79%), 4800-7500 (13%)		0.16
SLS	-----	-----	-----	-----	1.4	

* Initial solution conditions were temperature (T_{soln}), 30°C; preheater temperature ($T_{\text{preheater}}$), 60°C; pressure drop (ΔP), 345 bar; flow rate, 2.5 mL/min; and stabilizing bath temperature (T_{bath}), 25°C. Conc indicates concentration; max sol, maximum solubility; RESAS, rapid expansion from supercritical to aqueous solution; and surf, surfactant.

†1.0% (wt/wt)

Table 3. Compositions of the Various Phospholipid Surfactant Systems Used in This Study

Designation	Components	Concentrations % (wt/wt)	Vesicle Size Mean (nm)	Vesicle Size Range (nm)
Phospholipid A	Lipoid E80/ Tween 80/ Mannitol	10/2/5.5 1/0.2/0.55	39.6 35.1	20-50 10-50
Phospholipid B	Phospholipon 100H/ Tween 80/ Mannitol	2/2/5.5	90.2	70-280
Phospholipid C	Phospholipon 100H/ Myrj52/ Sodium Deoxycholate/ Mannitol	2/1/0.25/5.5	131.2	60-360
Phospholipid D	Phospholipon 100H/ Myrj52/ Mannitol	2/2/5.5	140.5	70-440

made with Lipoid E80 had starting vesicle sizes of 10 to 50 nm, while those made with Phospholipon 100H were slightly larger, as confirmed by DLS. The nonionic surfactants can act to make the vesicle bilayer more fluid,³⁰ facilitating transport across the bilayer. All of these systems included mannitol to enhance vesicle stability, and also to act as a cryoprotectant to prevent loss of structure during lyophilization.³¹⁻³³ Sodium deoxycholate was utilized in system "C" to supplement the steric stabilization with electrostatic stabilization.

Results are shown in Table 4 for the various phospholipid-based surfactant systems. The table shows the final concentration of drug in the suspension as measured by HPLC, the mean particle size, the particle size distribution (with the relative percentage of particles found in each peak for multiphase distributions), and the drug/surfactant ratio for the equilibrium solubility as well as at the final suspension concentration. In order to be able to distinguish between vesicles and particles in the final solutions, a few control experiments were performed. First, in the range of 10°C to 50°C, it was found that the size of the SUVs does not

Table 4. Effect of Surfactant Type on Cyclosporine Microparticles Prepared by RESAS for a Stabilizing Solution Bath Temperature of 25°C*

Phospholipid Surfactant Composition	Drug Conc (mg/mL)	Yield % (wt/wt)	Particle Mean (nm)	Particle Size Distribution (nm)	Drug/Surf Ratio (g/g)
A [†]	6.5	93	220	30-50 (4%), 100-160 (66%), 330-560 (30%)	0.54
A [†]	6.9	100	220	80-120 (32%), 250-300 (68%)	0.58
B	4.6	54	660	80-420 (91%), 3300-6300 (9%)	0.12
B	5.1	62	640	80-420 (93%), 5100-7100 (7%)	0.13
C	4.1	26	2740	140-300 (16%), 720-1740 (60%), 7500-10 000 (34%)	0.13
C	4.2	34	4110	280-470 (56%), 7700-10 000 (44%)	0.13
D	7.7	63	390	150-200 (54%), 560-750 (46%)	0.19
D	6.9	59	460	110-180 (54%), 320-560 (27%), 1180-2060 (19%)	0.17

* Initial solution conditions were temperature (T_{soln}), 30°C; preheater temperature ($T_{\text{preheater}}$), 60°C; pressure drop (ΔP), 345 bar; flow rate, 2.5 mL/min; and stabilizing bath temperature (T_{bath}), 25°C. Conc indicates concentration; RESAS, rapid expansion from supercritical to aqueous solution; and surf, surfactant.

[†]1.0% Lipoid E80/ 0.2% Tween 80/ 0.55% Mannitol (wt/wt); $T_{\text{bath}} = 45.0^\circ\text{C}$

change when measured by DLS. Second, if pure CO₂ is sprayed into the solution for a time similar to that in the rest of the experiments (up to an hour), there is also no change in the vesicle size even with the resulting change in pH from 7 to 3. pH is known to affect vesicles by increasing DPPC hydrolysis rates, and pH gradients have been utilized in the formation of SUVs.³⁴ Therefore, it is reasonable to believe that any change in particle size distribution as measured by DLS after RESAS with a drug would correspond to the presence of stabilized drug particles.

The low-concentration solution A was able to stabilize cyclosporine particles at concentrations of ~7 mg/mL (~0.6 drug:surfactant ratio) with mean diameters of 220 to 230 nm and relatively narrow size distributions as seen in Table 4. The distributions are bimodal. The smaller peak is somewhat larger than for the initial drug-free vesicles, suggesting that the drug induces modest aggregation of these vesicles. The larger peak is 5 to 10 times the size of the initial vesicles. It is likely that most of the drug is contained in these larger aggregates. The drug/surfactant ratios are over 20 times the equilibrium solubility in the vesicles. While the

examples discussed here were made with the lower concentration of formulation A, these suspensions tended to be less stable (discussed in more detail later) and were incapable of stabilizing drug payloads up to 50 mg/mL. Therefore, the majority of the rest of the work detailed herein was conducted using the higher concentration of formulation A.

Figure 4 shows transmission electron micrographs (TEMs) taken of several of the suspensions formed in this study. Figure 4A shows the initial empty SUVs of phospholipid formulation A before RESAS, with particle sizes that are between 10 and 80 nm. Upon exposure of the aqueous vesicles to CO₂, there was no noticeable change in the TEM micrographs. Note that in some regions, the vesicles associate loosely to form aggregates. Also, the vesicle shape appears distorted from a spherical shape. Some distortion can occur due to collapse or close packing of the vesicles upon water evaporation.^{27,34} The negative staining technique can be used to observe multilamellar structures (MLVs), if present. MLVs could be seen (not shown) before homogenization to form SUVs. Figure 4B shows the particles at a drug:surfactant ratio of 0.15, showing both the large stabi-

lized drug particles (~200 nm) as well as small vesicles. The DLS results showed particles as large as 400 nm, so there may be some aggregation occurring within the suspension between the larger drug particles and the remaining SUVs.

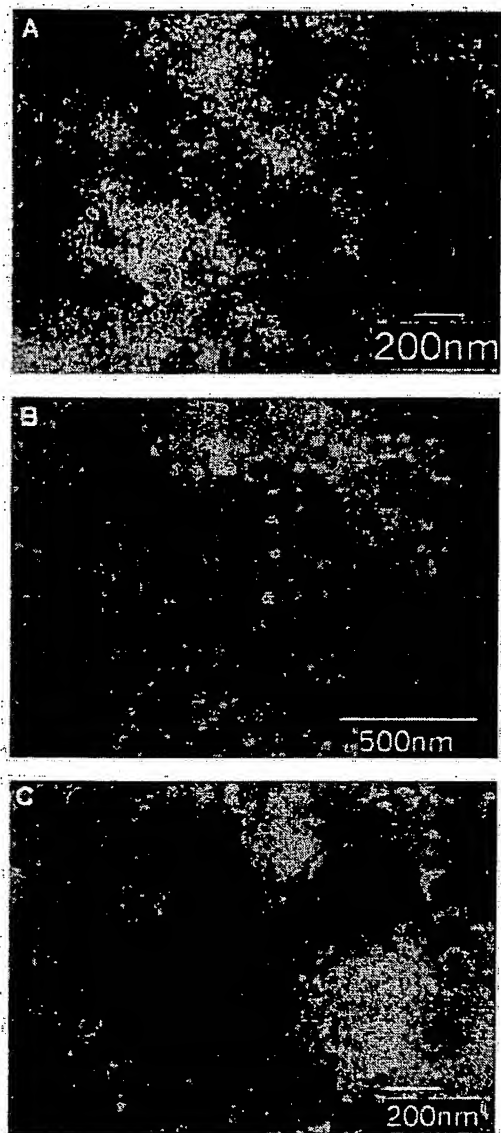


Figure 4. TEM micrographs of (A) initial SUVs made from phospholipid formulation A before RESAS, (B) drug-loaded vesicles of phospholipid formulation A at a concentration of 17.6 mg/mL and stabilizing solution temperature of 31.1°C; operating conditions: $T_{\text{soln}} = 30^{\circ}\text{C}$, $T_{\text{pre}} = 60^{\circ}\text{C}$, $\Delta P = 345$ bar, $T_{\text{bath}} = 45^{\circ}\text{C}$, solution flow rate = 2.5 mL/min, (C) drug-loaded vesicles of phospholipid formulation A at a concentration of 54.0 mg/mL and stabilizing solution temperature of 50.3°C; operating conditions: $T_{\text{soln}} = 30^{\circ}\text{C}$, $T_{\text{pre}} = 60^{\circ}\text{C}$, $\Delta P = 345$ bar, $T_{\text{bath}} = 80^{\circ}\text{C}$, solution flow rate = 2.5 mL/min.

Unfortunately, with this staining technique, we were unable to visualize the monolayer of surfactant on the particles, as the stain simply deposits around the outside of the particle. Also, we saw no evidence of successive bilayers surrounding the particles. While it was somewhat difficult to distinguish between drug particles and vesicles, the larger objects seen in this micrograph never appeared during inspection of phospholipid vesicles with no drug. Also, the entities we believe to be the drug particles have highly irregular surfaces, which is another characteristic not observed with unloaded vesicles. Figure 4C shows the particles formed at a high drug:surfactant ratio of 0.45, depicting very few small vesicles remaining with numerous large stabilized drug particles, approaching 300 nm. The DLS results in this case indicated 3 size ranges (40 to 60, 100 to 200, and 500 to 920) with the bulk of the material present in the last peak. This distribution again suggests that some of the particles in the TEM associate or aggregate.

The particle diameters of the phospholipid-stabilized suspensions were markedly lower than those produced with micellar-forming surfactants for similar surfactant concentrations and drug/surfactant ratios. To stabilize such small particles, the surfactant must be able to rapidly adsorb onto the surfaces of the precipitating particles in order to hinder particle growth in the jet. Since the bulk of the surfactant is now in the structure of a vesicle, the stabilization mechanism may be expected to be different than for the micelle-forming surfactants in Table 2. In the case of vesicles, the aggregation number of surfactant is much larger than for micelles. Thus, in a single vesicle the local concentration of surfactant that can coat a growing drug particle is higher than for a single micelle. The preferred curvature of the surfactant is more favorable for vesicles than micelles. For vesicles, the interface with water is less curved than for the much smaller micelles and will more closely match that of the drug particles. The better match in curvature may be expected to favor particle stability. The growing drug nuclei may collide with a bilayer of the vesicle and dissolve. The presence of the nonionic surfactant Tween 80 can aid the transport through the bilayer. Since vesicles tend to be relatively stable, growth of drug particles by collision/coagulation may be expected to be minimized. Thus, the particle may be expected to grow mostly due to a condensation mechanism within the bilayer as additional drug nuclei enter, although detailed studies would be needed to quantify this mechanism. When the drug particle size becomes large enough, it can disrupt the vesicle structure and essentially cause an “unzipping” of the bilayer as the vesicle breaks. The rearrangement of the surfactant may be expected, leaving a monolayer of the phospholipid on the particle surface with the polar heads solvated by water. SUVs may then become loosely associated with the hydrophilic groups on the outside surface of this monolayer. Previous

work has demonstrated the ability of phospholipid surfactants to adsorb on a hydrophobic surface as a monolayer.¹

Unfortunately, in all the cases where Phospholipon 100H was used, the solutions foamed extensively and quickly became very viscous or, as in the case of solution C, even gelled during the spray. Changing the temperature of these solutions from 25°C to 75°C had no observable or measurable effect on the particles collected or the nature of the solution during RESAS. When the solution becomes too thick, any further spraying yields large particles, as the surfactant can no longer diffuse to the particle surfaces as they precipitate and grow. In the case of phospholipid solution B, particles were stabilized with mean diameters from 370 to 660 nm with broad distributions from 30 nm to 7 μ m. Phospholipid solution C quickly gelled during the spray, yielding the largest particles of any of the phospholipid combinations with approximate mean particle sizes of 2 to 4 μ m and broad size distributions. Phospholipid solution D stabilized particles moderately well, with mean sizes of 290 to 460 nm and particles ranging from 70 nm to 3 μ m. Solutions B, C, and D also each had low trapping yields as the solutions became too viscous. It is likely that the difference between the performance of Lipoid E80 and Phospholipon 100H lies in the source (egg vs soybean), and resulting differences in impurities. Since phospholipid solution A produced the smallest particles, it will be the focus of the experiments in later sections concerning the effects of temperature, drug concentration, etc.

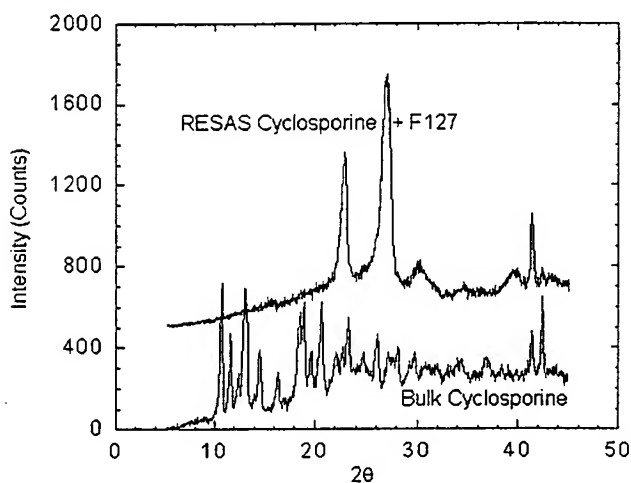


Figure 5. X-ray diffraction patterns for bulk cyclosporine (bottom) and cyclosporine processed by RESAS and stabilized by Pluronic F127 (drug/surfactant ratio = 0.2).

In Figure 5, we show the x-ray diffraction patterns for cyclosporine before RESAS (lower curve), and for cyclosporine stabilized by Pluronic F127 (upper curve). Prior to processing, the cyclosporine is crystalline, showing multiple sharp

peaks. After processing by RESAS and stabilization by F127, the cyclosporine crystal peaks have disappeared, suggesting the drug is now trapped in an amorphous state. The 2 large peaks seen are due to the surfactant, Pluronic F127. Since the entire sample produced with F127 was dried and analyzed (including the large particles), and the entire sample was amorphous, it is not unreasonable to assume that the smaller drug particles trapped with the other surfactant systems could also be amorphous.

Effect of Suspension Concentration

Tables 5-7 illustrate the effect of drug concentration, which is a function of spray time, on particle size for bath temperatures of 25°C, 45°C, and 80°C, respectively. In all of the experiments, the stabilizing solution was phospholipid formulation A at the higher concentration levels, as shown in Table 3. The starting vesicle size in the solution, as listed in Table 3, was 10 to 50 nm, and they are likely SUVs. Upon examination with TEM, the vesicles appeared similar to those shown in Figure 4A. Tables 5-7 show the actual stabilizing solution temperature measured within the separatory funnel in addition to the related entries found in Table 4.

Table 5 shows the particle size for a bath temperature of 25°C. At a drug concentration of ~20 mg/mL, particles collected had a mean diameter of 500 to 650 nm with sizes ranging from 70 nm to 2 μ m. At ~40 mg/mL, the mean particle size increased to 730 nm with very broad distributions. The average particle size grew by as much as 50% when doubling the drug concentration. A further increase in drug concentration to 46 mg/mL produced a substantial increase in particles larger than 1 μ m, indicating loss of stabilization against aggregation.

In Table 5 versus Table 6 the only difference in experimental conditions was the stabilizing solution bath temperature, which was 45°C. Initially, at a drug:surfactant ratio of ~0.1 (roughly 2 times the equilibrium solubility), the particle mean diameters were only 160 to 180 nm with narrow size distributions. At a drug:surfactant ratio of ~0.15, the particle mean increased to 260 to 290 nm, and at 0.25, it reached 310 to 390 nm with a slightly broader size distribution. In all cases, the particles were much smaller than those produced with Tween 80.

In Table 7, the stabilizing solution bath temperature was 80°C. For a given drug loading, each of the properties of the particles was similar to that for a bath temperature of 45°C. In all 3 cases, the particle size increased substantially with drug concentration, which increases with spray time. Figure 6 demonstrates the trends in particle growth more clearly. For the 2 higher stabilizing solution temperatures, the particle size increases approximately linearly with drug concentration, with only a small increase in size with temperature.

Table 5. Effect of Suspension Concentration on Cyclosporine Microparticles Prepared by RESAS for a Stabilizing Solution Bath Temperature of 25°C*

Stabilizing Solution Temp °C	Drug Conc (mg/mL)	Yield % (wt/wt)	Particle Mean (nm)	Particle Size Distribution (nm)	Drug/Surf Ratio (g/g)
14	18.4	89	650	70-140 (28%), 240-450 (36%), 1000-1900 (36%)	0.15
13.3	19.4	96	500	90-190 (45%), 530-1260 (55%)	0.16
14.8	24.2	110	630	120-210 (47%), 760-1440 (53%)	0.20
15.8	39.0	110	730	50-80 (4%), 160-290 (33%), 750-1500 (63%)	0.33
13.7	39.8	80	960	80-160 (14%), 480-910 (74%), 2700-5200 (12%)	0.33
13.6	45.9	94	1700	80-160 (12%), 500-1150 (74%), 5000-10000 (14%)	0.38

* Initial solution conditions were temperature (T_{soln}), 30°C; preheater temperature ($T_{\text{preheater}}$), 60°C; pressure drop (ΔP), 345 bar; flow rate, 2.5 mL/min; and stabilizing bath temperature (T_{bath}), 25°C. Conc indicates concentration; RESAS, rapid expansion from supercritical to aqueous solution; surf, surfactant; and temp, temperature.

Table 6. Effect of Suspension Concentration on Cyclosporine Microparticles Prepared by RESAS for a Stabilizing Solution Bath Temperature of 45°C*

Stabilizing Solution Temp °C	Drug Conc (mg/mL)	Yield % (wt/wt)	Particle Mean (nm)	Particle Size Distribution (nm)	Drug/Surf Ratio (g/g)
32	10.3	70	160	80-110 (67%), 260-350 (33%)	0.09
33	13.0	87	180	80-110 (47%), 220-290 (53%)	0.11
31.2	15.8	89	280	40-70 (6%), 140-220 (67%), 450-760 (27%)	0.13
29.8	17.5	95	290	110-160 (44%), 370-470 (56%)	0.15
31.1	17.6	93	260	90-120 (33%), 290-400 (67%)	0.15
30.3	26.2	72	380	40-80 (10%), 200-370 (71%), 690-1100 (19%)	0.22
31.2	24.4	74	310	40-100 (13%), 200-520 (87%)	0.20
30.5	31.7	83	390	30-50 (2%), 140-230 (44%), 430-820 (54%)	0.26

* Initial solution conditions were temperature (T_{soln}), 30°C; preheater temperature ($T_{\text{preheater}}$), 60°C; pressure drop (ΔP), 345 bar; flow rate, 2.5 mL/min; and stabilizing bath temperature (T_{bath}), 45°C. Conc indicates concentration; RESAS, rapid expansion from supercritical to aqueous solution; surf, surfactant; and temp, temperature.

Table 7. Effect of Suspension Concentration on Cyclosporine Microparticles Prepared by RESAS for a Stabilizing Solution Bath Temperature of 80°C*

Stabilizing Solution Temp °C	Drug Conc (mg/mL)	Yield % (wt/wt)	Particle Mean (nm)	Particle Size Distribution (nm)	Drug/Surf Ratio (g/g)
56.8	12.6	88	220	60-90 (45%), 270-380 (55%)	0.11
54.3	14.8	88	230	60-90 (40%), 290-390 (60%)	0.12
57.2	17.6	72	320	60-100 (28%), 310-600 (72%)	0.15
53.7	23.8	62	390	80-120 (34%), 440-650 (66%)	0.20
53.8	27.0	72	460	100-140 (31%), 500-750 (69%)	0.23
52.7	39.8	99	480	50-100 (48%), 170-360 (18%), 860-1770 (34%)	0.33
50.3	54.0	139	500	40-60 (26%), 100-200 (11%), 500-920 (63%)	0.45

*Initial solution conditions were temperature (T_{soln}), 30°C; preheater temperature ($T_{\text{preheater}}$), 60°C; pressure drop (ΔP), 345 bar; flow rate, 2.5 mL/min; and stabilizing bath temperature (T_{bath}), 80°C. Conc indicates concentration; RESAS, rapid expansion from supercritical to aqueous solution; surf, surfactant; and temp, temperature.

For a temperature of 14°C, the size is larger by a factor of about 2, and the scatter about the linear correlation with drug concentration is much larger.

Several factors may be expected to cause the increase in particle size with drug concentration. The first factor is surfactant depletion in the aqueous solution as the SUVs coat the particles and form drug-surfactant aggregates. As the spray time and drug concentration increase, fewer of the initial SUVs are available for stabilizing incoming particles. Another factor is that the particle collision rate increases approximately with the square of the particle concentration. In addition, an increase in the drug concentration raises the drug/surfactant ratio in the aqueous solution. The resulting increase in total drug surface area and decrease in surfactant coverage of this drug surface area may lead to greater aggregation of the surfactant-coated drug particles. Another factor is simply the greater time for particle growth due to shear-induced aggregation caused by the flowing CO₂. Once removed from the process, however, the drug suspensions were extremely stable with little change in particle size measurable by DLS even after weeks of storage, as discussed in greater detail below.

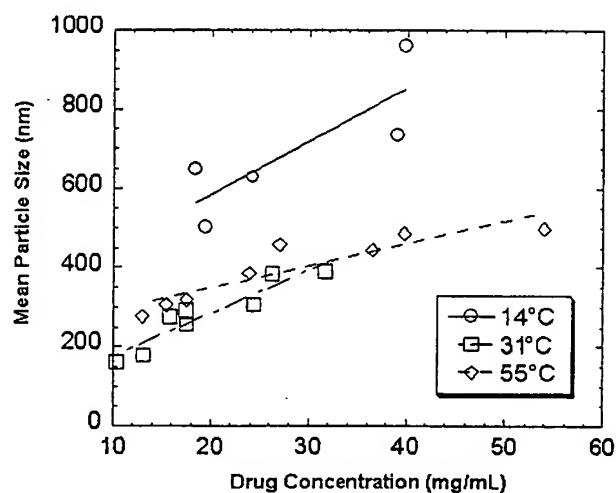


Figure 6. Effect of drug concentration and stabilizing solution temperature on cyclosporine particles produced by RESAS with phospholipid solution A as the stabilizer. T_{soln} = 30°C; $T_{\text{pre}} = 60^\circ\text{C}$; $\Delta P = 345$ bar; $T_{\text{bath}} = 25^\circ\text{C}, 45^\circ\text{C}, 80^\circ\text{C}$; solution flow rate = 2.5 mL/min.

Effect of Stabilizing Solution Temperature

The ability for the phospholipid-based surfactants to stabilize the particles may be expected to depend upon the temperature of the medium within the receiving vessel. Most phospholipid vesicles exhibit a transition temperature from a rigid gel-like state to a fluid liquid-crystalline state. As temperature increases, the phospholipid chains within the vesicles go from a very rigid, ordered state, to one that is more flexible and can allow diffusion across the bilayer. The rigidity of the phospholipid tails, and hence the vesicles, could affect the surfactant's ability to rearrange in order to stabilize the drug particles as they precipitate. The effect of lipid chain melting behavior, and hence the lipid's ability to orient at the surface of emulsion droplets has been studied.²⁷ Dry Lipoid E80 powder was analyzed by DSC to determine the chain melting point, as shown in Figure 7. The melting transition appears slightly above room temperature, at 24°C to 29°C. While the location of the transition agrees with previous studies,²⁷ the transition enthalpy is lower here. This difference is likely due to adsorbed water. When 10% (wt/wt) of Lipoid E80 was added to water, however, the DSC thermogram was dominated by the water melting peak, and since the transition enthalpy was already low, it could no longer be detected. This effect has been seen previously.²⁷ Also, the fact that it is hard to detect a clear transition in solution is not unexpected for lipid samples that are not made of pure components or that have additives such as cholesterol.³⁵ While the presence of Tween 80, mannitol, and even the drug in the vesicles would be expected to further broaden/shift the transition point,^{27,35-40} this effect could unfortunately not be demonstrated with this system.

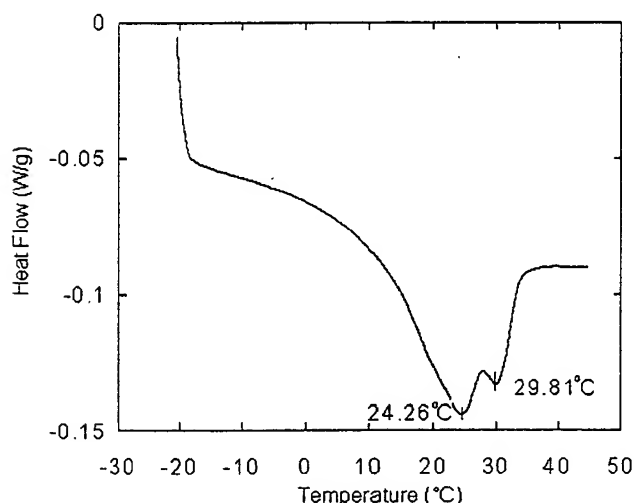


Figure 7. DSC thermogram of bulk Lipoid E80.

Note that in each of the tables, the actual stabilizing solution temperature near the nozzle is significantly lower than that

of the water bath used to heat the medium. This cooling is due to the expanding CO₂ gas. As seen in Figure 4, the particle sizes measured when the stabilizing solution temperature was above 30°C were nearly the same at similar drug loadings, regardless of the stabilizing solution bath temperature. However, a large increase in particle size was noted for the cases in which the stabilizing solution was only 14°C. It is likely that at this temperature the phospholipid chains are too rigid to rearrange and stabilize the growing particles as rapidly as at the higher temperatures. The increase in the viscosity of water at locally low temperatures could further inhibit diffusion and rearrangement of surfactant. There could also be an effect of locally colder temperatures in the vicinity of the nozzle tip decreasing supersaturation based on the phase diagram,²⁵ slowing particle nucleation and producing larger particles. This supersaturation effect is discussed in greater detail in the next section on the effect of preheater temperature for a constant stabilizing solution temperature.

Effect of Preheater Temperature

Cyclosporine becomes less soluble in CO₂ as temperature increases.²⁵ Therefore, it is expected that at higher preheater temperatures, the depressurization in the nozzle will cause the solution to pass through the phase boundary more quickly than at lower temperatures, leading to higher levels of supersaturation.^{15,25,41} The greater supersaturation, in turn, leads to higher rates of nucleation, which would lead to the formation of smaller particles, if aggregation is controlled.

Table 8 illustrates the effects of the preheater temperature on the cyclosporine particle size. The first 2 entries show the results through a nozzle at a flow rate of 0.88 mL/min at a change in pressure (ΔP) of 345 bar. At a low preheater temperature of 30°C, the particles produced at this flow rate were larger than 10 μ m and quickly settled out, leaving only drug solubilized in the SUVs. With a bath temperature of only 30°C, the stabilizing solution temperature became too cold for adequate stabilization as discussed above. At a higher preheater temperature of 60°C and at a drug:surfactant ratio of 0.24, the particles had a mean size of 530 nm. The higher preheater temperature resulted in less local cooling for this nozzle flow rate and allowed the stabilization of particles.

In the remainder of the results in Table 8, the solution flow rate was 2.5 mL/min at the same ΔP of 345 bar. As the preheater temperature was changed from 30°C to 80°C, the mean particle size decreased. The drug:surfactant ratio was fairly constant, especially for the 2 higher preheater temperatures. Notice that the preheater temperature had essentially no effect on the stabilizing solution temperature because of the efficient heat transfer in the nozzle and the jet,

Table 8. Effect of Preheater Temperature on Cyclosporine Microparticles Prepared by RESAS of a 1.0% (wt/wt) solution into 10.0 mL of phospholipid mixture A*

T_{pre} °C	Stabilizing Solution Temp °C	Drug Conc (mg/mL)	Yield % (wt/wt)	Particle Mean (nm)	Particle Size Distribution (nm)	Drug/Surf Ratio (g/g)
30 [†]	22.0	2.5	30		30-130, >10 μ m	0.02
60 [†]	21.2	24.0	80	530	190-290 (82%), 1500-2500 (18%)	0.24
30	32.1	27.8	100	400	50-240 (40%), 440-720 (60%)	0.23
30	31.7	19.3	82	380	90-170 (15%), 270-590 (85%)	0.16
60	32.2	14.3	98	280	40-180 (68%), 420-770 (32%)	0.12
60	31.8	11.9	84	320	110-150 (41%), 400-540 (59%)	0.10
80	31.6	11.7	79	110	70-100 (90%), 280-380 (10%)	0.10
80	32.0	13.1	78	190	20-140 (70%), 280-380 (30%)	0.11

*Initial solution conditions were temperature (T_{soln}), 30°C; pressure drop (ΔP), 345 bar; flow rate, 2.5 mL/min; and stabilizing bath temperature (T_{bath}), 45°C. Conc indicates concentration; RESAS, rapid expansion from supercritical to aqueous solution; surf, surfactant; and temp, temperature; and T_{pre} preheater temperature.

[†] T_{soln} = 30°C; T_{bath} = 40°C; ΔP = 345 bar; solution flow rate = 0.88 mL/min.

and the large change in latent heat for compressed CO₂ relative to the sensible heat. Consequently, the rigidity of the phospholipids in the aqueous solutions was essentially constant. Thus, the decrease in particle size is most likely due to the faster nucleation rate produced by the higher supersaturation at the higher preheater temperatures.

Effect of Nozzle Size

Table 8 also illustrates the effect of nozzle size on the particle size. Two crimped nozzles were compared with similar operating pressures, ΔP = 345 bar, but with flow rates of 0.88 mL/min and 2.5 mL/min. The greater restriction for the slower flow rate nozzle indicates that the crimping and filing process produced a smaller elliptical orifice. The smaller, low flow rate nozzle appears to produce larger particles than the high flow rate nozzle. While smaller droplet sizes would be expected with the smaller nozzle due to more intense atomization, the dominating factor on particle stability in this system appears to be the conditions of the stabilizing solution. At the lower flow rate, the jet is less forceful and creates less mixing/turbulence within the stabilizing solution. The weaker mixing is evident in the lower local temperature within the stabilizing solution. At the higher flow

rates, the solution is agitated more intensely, and heat is transferred throughout the fluid within the separatory funnel more efficiently, producing a higher local temperature. Since the lower flow rate nozzle creates less turbulence, the particles and surfactant are not mixed as well, leading to less-effective stabilization. Also, the particles are not carried away from the jet as quickly, allowing for potentially more collisions prior to complete stabilization. In the future, this limitation could be overcome by stirring the solution.

The use of a much larger 50- μ m id \times 10-cm long straight capillary nozzle in RESAS led to low yields (<35%) and the formation of >10 μ m particles. Also, this nozzle tended to plug easily as compared with the crimped nozzle design, which never plugged during a spray. In the case of the capillary nozzle, the depressurization profile occurs over the entire length of the nozzle, resulting in some particle nucleation and growth within the nozzle where it cannot be stabilized.

Long-term Stability

For all of the samples produced, initial particle size measurements were made within 24 hours of the RESAS spray. Also, the stability of the particle size after 1 month of stor-

Table 9. Suspension Stability After 1 Month of Storage at 4°C for Phospholipid Mixture A 10%/ 2%/ 5.5% (wt/wt) Lipoid E80/Tween 80/mannitol

Original Mean (nm)	Mean After 1 Month (nm)	Distribution After 1 Month (nm)	% Change
260	320	30-50 (25%), 120-190 (16%), 410-710 (59%)	20
220	750*	50-230 (17%), 660-1200 (83%)	240
1700	720	100-160 (10%), 650-1000 (90%)	-40
390	700	80-140 (29%), 200-300 (6%), 820-1230 (65%)	80
460	560	50-240 (47%), 560-1780 (53%)	20
400	740	60-90 (41%), 170-350 (16%), 1200-2000 (43%)	85
320	420	50-150 (46%), 220-410 (15%), 680-1120 (39%)	30

*1.0%/0.2%/0.55% (wt/wt) Lipoid E80/Tween 80/mannitol.

age at 4°C and under nitrogen was examined as shown in Table 9. Each sample was gently inverted 5 to 10 times and then allowed to sit for 1 hour prior to analysis. For each of the nonphospholipid surfactants, the particles settled completely and caked at the bottom of the vial. These samples were not redispersible, even by sonication. The samples produced by RESAS with phospholipid solution A had good stability, with no settling observed for most of the suspensions without shaking. Even in cases where there was some settling present, the samples were easily redispersed by the gentle inversions. Most of the samples experienced some growth, or small broadening in the size distribution. In one case, the particle size grew considerably during the 1-month period (likely because of the very high drug:surfactant ratio (>0.5) in this system due to the low amount of surfactant) and also showed instability in the form of settling. In the case in which the particle size appears to decrease, it is likely that some of the larger particles have settled out (not visible to eye) and thus were simply not captured in the sample analyzed by DLS.

In summary, many of the size distributions were bimodal, according to DLS measurements and TEM, consisting of vesicles with very low drug concentrations and drug particle aggregates stabilized with vesicles. As the local temperature of the aqueous stabilizing solution is increased above 25°C, the vesicles become less rigid and stabilize the drug particles

more effectively, leading to smaller particles. For a given stabilizing solution temperature, the particle size also decreases with an increase in preheater temperature due to greater supersaturation in the nozzle and more rapid nucleation. High drug loadings in the aqueous suspensions, as high as 54 mg/mL, could be achieved with particle sizes of ~500 nm. This concentration is ~10 times the equilibrium solubility in the aqueous surfactant solution. For Pluronic F127 as a stabilizer, the particles are stabilized rapidly enough to trap the drug in an amorphous state. Long-term stability studies of the suspensions stored at 4°C indicate only modest particle growth over 1 month. The particle sizes in RESAS are much smaller than those produced by RESS as a result of the stabilization provided by the surfactant. The sizes are comparable with those produced by homogenization.

CONCLUSION

Phospholipid vesicles mixed with nonionic surfactants stabilize cyclosporine particles produced by RESAS with mean diameters as small as 200 to 300 nm. This size range is several hundred nanometers smaller than produced by RESAS for particles stabilized by Tween 80. The high drug loadings in the aqueous suspensions, reaching 50 mg/mL (~10 times greater than the equilibrium solubility); the small particle

sizes; and the long-term stability make this process attractive for development.

ACKNOWLEDGEMENTS

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